CAENORHABDITIS ELEGANS HOST CELL FACTOR 1 MODULATES ORGANISMAL LONGEVITY AND STRESS RESPONSES VIA COORDINATED INTERACTIONS WITH MULTIPLE NUCLEAR TRANSCRIPTION FACTORS AND REGULATORS

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C. ELEGANS HOST CELL FACTOR 1 MODULATES ORGANISMAL LONGEVITY AND STRESS RESPONSES VIA COORDINATED INTERACTIONS WITH MULTIPLE NUCLEAR TRANSCRIPTION FACTORS AND REGULATORS

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Aging is a complex process influenced by the environment and genotype. Numerous conserved genetic pathways and factors have been identified as key mediators of lifespan and stress responses in the nematode *C. elegans.* Host cell factor-1 (HCF-1) is a longevity and stress response modulator in worms. Mammalian HCF-1 is a vital transcriptional regulator which scaffolds diverse transcriptional regulatory complexes and controls gene expression. In *C. elegans,* HCF-1 is a repressor of the critical longevity determinant DAF-16, the homolog of mammalian FOXO transcription factors. The molecular partners of HCF-1 and the mechanisms whereby it modulates lifespan and stress responses have not been fully elucidated.

My work implicated HCF-1 as a critical player in the regulatory mechanism linking DAF-16 and its coactivator SIR-2.1 in worms. Genetic analyses revealed that *hcf-1* acts downstream of *sir-2.1* to influence lifespan and oxidative stress response. Gene expression profiling uncovered a striking 80% overlap between the HCF-1- and SIR-2.1-regulated DAF-16 target genes. Subsequent GO-term analyses of HCF-1 and SIR-2.1-coregulated DAF-16 targets suggested that HCF-1 and SIR-2.1 together regulate specific aspects of DAF-16-mediated transcription important for aging and stress responses. My findings uncover a novel interaction between the key longevity determinants SIR-2.1 and HCF-1, and provide new insights into the complex regulation of DAF-16.

SKN-1 transcription factor is an evolutionarily conserved protector against oxidative and xenobiotic stress and is a well-established pro-longevity factor. I demonstrated that SKN-1 contributes to the enhanced oxidative stress resistance incurred by *hcf-1* inactivation in a manner parallel to DAF-16. This functional interaction between HCF-1 and SKN-1 specifically occurs under excessive oxidant stress as SKN-1 is dispensable for the thermotolerance and long lifespan of *hcf-1* mutants. HCF-1 represses the activation of SKN-1 to inhibit SKN-1 target genes involved in cellular detoxification pathways. To control SKN-1 activity, HCF-1 prevents nuclear accumulation of SKN-1 in response to oxidative stress. My findings reveal a new, context-specific regulatory relationship between the stress-response factors HCF-1 and SKN-1.

Given that HCF-1, DAF-16, SIR-2.1, and SKN-1 are functionally conserved between *C. elegans* and mammals, my findings have important implications for the regulation of mammalian counterparts of these factors by HCF proteins.

BIOGRAPHICAL SKETCH

Gizem Rizki was born in the beautiful coastal town of Kyrenia in the small island of Cyprus in the Mediterranean Sea. Gizem's school and social life began quite early in life when she started daycare at age 40 days. She has not left academics since. With the encouragement (and sometimes push) of her mom, Gizem spent most of her childhood and teenage years exploring and engaging in many extracurricular activities besides school. She was part of the school basketball team in elementary and high school, track team in elementary school, played tennis, went to ballet, horsebackriding, taekwondo, painting, guitar, and piano lessons. Due to her involvement in many diverse activities, Gizem developed multitasking skills early on in life. Her passion for music and her extraordinary piano teacher allowed her to pursue and successfully pass many England Royal School of Music piano examinations starting from age 5 to age 17. Throughout high school, Gizem's life was focused upon studying for GCE, SAT, and school exams to apply for a prestigious merit-based AMIDEAST-CASP scholarship given to Cypriot students to pursue an undergraduate education in the U.S.A. Her hard work paid off when she was granted a full CASP scholarship. She was subsequently accepted to the University of California at Berkeley and her U.S.A. adventure began in 1998. Gizem completed her BA degree with honors in Molecular and Cell Biology at Berkeley in 2002. It is here that Gizem's interest in research peaked as she participated in undergraduate research in Dr. Paul D. Kaufman's laboratory. After college, she worked at UC-San Francisco as a research assistant in Dr. Jacquelyn J. Maher's lab for one year before returning home to Cyprus and working as a research associate at Cyprus Institute of Neurology and Genetics. After two years, Gizem decided to embark on yet another journey and continue her education by doing a PhD. She returned to the U.S.A. to begin graduate school at Cornell University, studying under Dr. Sylvia Lee to investigate the genetic and molecular mechanisms of aging. Here, she met amazing friends one of whom turned out to be her future husband, Dr. Aaron Plys. Despite spending the majority of her life

in the warm climates of the Mediterranean and California, Gizem bravely withstood the viciously cold Ithaca winters. Equipped with her enforced survival skills after 6 years doing a PhD and living in Ithaca, Gizem will embark on yet another (more sunny) journey when she returns home to Cyprus for one year, followed by postdoctoral training.

This document is dedicated to my mom, dad, sister, and husband, because of whose unending support and love I was able to complete this work. Sizi çok seviyorum.

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HCF-1	Host Cell Factor-1
DAF-16	Abnormal Dauer Formation-16
SIR-2.1	Silent information regulator-2.1
SKN-1	Skinhead Family member-1
DR	Dietary Restriction
CR	Caloric Restriction
TOR	Target of Rapamycin
IIS	Insulin/IGF-like Signaling
FOXO	Forkhead Box O transcription factor
RR-qPCR	Reverse Transcription coupled quantitative PCR
ctrl	Control
GO	Gene Ontology
O/E	Overexpression
GFP	Green Fluorescent Protein
MAPK	Mitogen activated protein kinase
HMT	Histone methyl transferase
SMK-1	Suppressor of MEK null homolog
HSF-1	Heat shock factor 1
CTBP-1	C-terminal binding protein 1

CHAPTER 1

INTRODUCTION

Progression of age is a major risk factor for the development of numerous age-related, life-threatening ailments such as cancer, diabetes, neurological disorders and heart disease leading to a marked decline in the quality of life. Understanding the detailed biology of the aging process is undoubtedly crucial for facilitating better treatments for age-associated diseases thus extending the length and improving the quality of life. For several decades, a large body of research has been focused on uncovering the underlying genetic and molecular determinants of aging and age-related diseases. The development of suitable model systems to allow for efficient and thorough studies of aging is essential. Due to its short lifespan of 2-3 weeks and its powerful genetic tools, the nematode *Caenorhabditis elegans (C. elegans)* serves as an exceptional organism for aging research. Indeed, *C. elegans* has been instrumental in identifying and characterizing genetic components that influence aging. Studies in worms have been successfully extended to complex mammalian organisms allowing for the identification of genetic factors that impact longevity in mammals. Mitochondrial alterations, caloric restriction, reproductive signals, translation initiation network, Target of Rapamycin (TOR) pathway, and insulin/IGF-1 like signaling are among the major pathways that modulate aging and stress responses (Kenyon, 2010). DAF-16/FOXO transcription factors are among the best characterized longevity factors and their increased activity leads to a longer lifespan and improved stress resistance in many organisms. Host Cell Factor 1 (HCF-1) is a highly conserved longevity determinant which acts as a corepressor of DAF-16 in *C. elegans* (Li *et al.*, 2008). As a highly conserved and dynamic transcription cofactor, HCF-1 represents a potential hub for coordinating and

regulating the activities of multiple longevity factors (Lu *et al.*, 1997; Gunther *et al.*, 2000; Lu and Misra, 2000; Wysocka *et al.*, 2003). Exploring the molecular partners of HCF-1 and understanding the mechanisms by which this novel longevity factor functions to modulate aging and stress responses will provide new insights into the basic biology of aging and will aid future therapeutic developments aiming to improve healthy aging and alleviate age-related diseases in humans.

1.1 Host Cell Factor 1

C. elegans Host Cell Factor-1 (HCF-1) belongs to a family of highly conserved HCF proteins. HCF homologs encompass characteristic N-terminal Kelch and C-terminal Fibronectin repeat domains necessary for protein-protein interactions (Wilson *et al.*, 2000; Izeta *et al.*, 2003a). There are two HCF counterparts in mammals, HCF-1 and HCF-2. So far, HCF-1 is the more extensively studied HCF family member in mammals and the cellular functions of HCF-2 remain largely unknown. Until recently, the functions of HCF-1 have been mainly characterized in mammalian cell culture systems. Human HCF-1(HsHCF-1) was originally identified as a binding partner of the Herpes Simplex Virus (HSV) VP16 transcription factor (Gerster and Roeder, 1988). Upon infection of mammalian cells, VP16 binds cellular transcriptional accessory proteins HCF-1 and Oct-1 to assemble a DNA-associated complex and initiate the expression of immediate early genes necessary for viral infection (Gerster and Roeder, 1988; Kristie *et al.*, 1989; Katan *et al.*, 1990; Xiao and Capone, 1990). Apart from participating in a VP16-mediated complex, HCF-1 normally serves as a transcriptional coregulator and scaffolds the assembly of transcription factor complexes to enhance or repress their transactivation properties. HCF1 carries out several different modes of regulation of transcription factor activities: 1) it antagonizes the interaction between a

transcription factor and its coactivator (Piluso *et al.*, 2002), 2) it represses or activates gene expression through recruiting and assembling appropriate transcription and chromatin modifying complexes at target gene promoters (Wysocka *et al.*, 2003; Tyagi *et al.*, 2007). To date, many different factors have been found to associate with HCF-1 pointing to the diverse cellular functions of this protein. CREB/ATF transcription factor Luman (Freiman and Herr, 1997; Lu *et al.*, 1997) and its inhibitor Zhangfei (Lu and Misra, 2000; Misra *et al.*, 2005), transcription factors GABP (Vogel and Kristie, 2000) and Sp1 (Gunther *et al.*, 2000), Peroxisome proliferator-activated receptor gamma coactivator PGC-1 (Lin *et al.*, 2002), protein phosphatase PP1 (Ajuh *et al.*, 2000), chromatin modifier complexes Sin3 histone deacetylase (HDAC), Set1/Ash2 histone methyltransferase (HMT) and Mixed-lineage leukemia (MLL) HMT (Wysocka *et al.*, 2003; Yokoyama *et al.*, 2004), and cell-cycle regulatory transcription factors Miz-1 (Piluso *et al.*, 2002) and E2F factors (Knez *et al.*, 2006; Tyagi *et al.*, 2007) are among many diverse HCF-1 interacting partners.

Most notably, mammalian HCF-1 is a key regulator of cell cycle progression and cellular proliferation. HCF-1 is required for many aspects of cellular growth and division in human cells: proper cytokinesis, progression through G1 phase; exit from mitosis (Goto *et al.*, 1997; Reilly and Herr, 2002; Julien and Herr, 2003). HCF-1 acts as a coregulator of E2F-mediated transcription of genes necessary for cell-cycle progression (Tyagi *et al.*, 2007). When associated with the transcriptional activator E2F1, HCF-1 recruits activating Set-1 histone H3 lysine 4 (H3K4) methyltransferase (HMT) and MLL chromatin modifying complexes to E2F-responsive promoters to induce gene expression. In contrast, HCF-1 selectively recruits repressive chromatin modifiers such as Sin3 histone deacetylase (HDAC) when bound by the cell-cycle gene repressor E2F4 (Tyagi *et al.*, 2007). Recently, HCF-1 has been shown to be involved in mouse embryonic development and stem cell maintenance via

associations with Ronin, a factor required for embryogenesis and maintenance of embryonic stem (ES) self-renewal capacity (Dejosez *et al.*, 2008). Overall, HCF-1 represents a dynamic transcription cofactor capable of engaging in diverse transcriptional regulatory complexes. Functions of mammalian HCF-1 apart from inducing the expression of HSV VP16-regulated viral genes and regulating the transcription of genes required for cell-cycle progression and embryonic stem cell proliferation has yet to be discovered.

C. elegans HCF-1 (CeHCF-1) shares extensive structural homology with its two mammalian counterparts, HCF-1 and HCF-2 (Lee and Herr, 2001). Although all three proteins encompass two highly conserved Kelch and Fibronectin repeat domains, both CeHCF-1 and mammalian HCF-2 lack several internal basic, acidic, and proteolytic cleavage domains present in mammalian HCF-1 (Figure 1.1). Despite the structural divergence between the worm and mammalian HCF-1 proteins, several important cellular functions are well-conserved between them. Even though all three factors can direct VP16-induced complex formation, only HCF-1 and CeHCF-1 are capable of promoting VP16-regulated transcription (Lee and Herr, 2001). Similar to HCF-1, CeHCF-1 also associates with the Zinc finger MYND domain-containing protein PDCD2 involved in programmed cell death (Scarr and Sharp, 2002). Both mammalian and worm HCF proteins are ubiquitously expressed, however only HCF-1 and CeHCF-1 are exclusively nuclear, whereas HCF2 displays dynamic localization patterns between cytoplasmic and nuclear compartments (Kristie *et al.*, 1995; Johnson *et al.*, 1999; Izeta *et al.*, 2003b; Li *et al.*, 2008). Mutation of *C. elegans* HCF-1 leads to embryonic lethality as well as mitotic and cytokinetic defects similar to those caused by the loss of mammalian HCF-1. Furthermore, HCF-1 regulates the phosphorylation of histone H3 in worms and mammals (Lee *et al.*, 2007). Collectively, the above observations highlight a strong functional conservation between *C. elegans* and mammalian HCF-1.

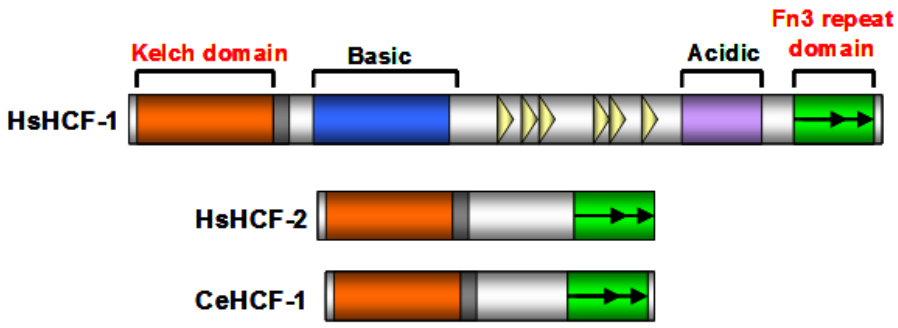


Figure 1.1. HCF proteins are highly conserved between *C. elegans* and mammals.

C. elegan HCF-1 shares greater than 50% sequence identity with the Kelch and Fibronectin 3 (Fn3) repeat domains present in mammalian HCF-1 and HCF-2 family members (Lee and Herr, 2001). Kelch and Fn3 repeat domains constitute critical protein-protein interaction regions that mediate associations of HCF-1 with its diverse molecular partners. Although the protein structure of *C. elegans* HCF-1 appears to more closely resemble that of mammalian HCF-2, it in fact shares many functional similarities with the mammalian HCF-1.

HCF-1 has an important role in aging in *C. elegans.* In a genome-wide RNAi screen, *hcf-1* was identified as a longevity determinant (Hamilton *et al.*, 2005). HCF-1 acts as a nuclear repressor of the highly conserved and extensively studied longevity factor DAF-16/FOXO. Inactivating *hcf-1* robustly extends lifespan and confers oxidative and heavy-metal stress resistance in a *daf-16*-dependent manner. In the nucleus, HCF-1 physically associates with DAF-16 and limits its access to a subset of target gene promoters (Li *et al.*, 2008). Interestingly, in regulating DAF-16, *hcf-1* may act in parallel to the insulin and germline signaling pathways, both of which are major modulators of DAF-16 activity (Li *et al.*, 2008). The exact mechanism by which HCF-1 inhibits DAF-16 and whether additional factors are involved is unknown. Considering that the main function of the mammalian HCF-1 is to assemble transcriptional regulatory complexes, it is reasonable to hypothesize that in *C. elegans* HCF-1 interacts with additional DAF-16 cofactors to assemble functional regulatory units to modulate DAF-16 activity.

1.2 DAF-16/FOXO longevity determinant (Figure 1.2)

C. elegans Abnormal *da*uer *f*ormation-*16* (*daf-16*) is the best characterized longevity determinant to date. *daf-16* was originally identified as a gene whose mutation inhibited formation of a stress-resistant developmental arrest state called dauer (Albert *et al.*, 1981). DAF-16 is a member of the evolutionarily conserved Forkhead Box Class O (FOXO) transcription factors (Lin *et al.*, 1997; Ogg *et al.*, 1997). DAF-16, as well as the *Drosophila,* mouse, and human FOXO transcription factors are all critical for longevity, metabolism, stress response, apoptosis, and cell proliferation (Kenyon *et al.*, 1993; Lin *et al.*, 1997; Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004; Pinkston *et al.*, 2006; Pinkston-Gosse and Kenyon, 2007; Arden, 2008; Kappeler *et al.*, 2008; Wang *et al.*, 2008; Willcox *et al.*, 2008; Li *et al.*, 2009; Yuan *et al.*, 2009), suggesting that the mechanisms underlying FOXOs' ability to

affect physiology are highly conserved across species. Indeed, much of our understanding of FOXO regulation comes from studies done on *C. elegans* DAF-16. DAF-16 selectively activates and represses the transcription of a large number of genes, cumulative action of which results in stress resistance, altered metabolic and developmental responses, increased immunity, and longevity (Honda and Honda, 2002; Lee *et al.*, 2003; McElwee *et al.*, 2003; Murphy *et al.*, 2003; Halaschek-Wiener *et al.*, 2005).

DAF-16 is justly referred to as a "master regulator" of longevity and physiology as it is an exceptionally dynamic transcription factor capable of integrating many environmental and cellular inputs and interacting with a wide variety of factors to initiate responses to upstream stimuli (Mukhopadhyay *et al.*, 2006). To integrate a large variety of cues and coordinate proper transcriptional responses to each, DAF-16 activity needs to be very tightly regulated at the level of post-translational modification, nuclear/cytoplasmic localization, association with co-regulators, and degradation. Additional layers of regulation through tissue-specific functions and different specialized isoforms have been implicated for DAF-16. Although ubiquitously expressed, DAF-16 activity in the intestine and neurons appears to be predominantly required for lifespan extension, suggesting that DAF-16 acts nonautonomously to coordinate aging and stress responses in the whole organism (Libina *et al.*, 2003). It has recently been reported that different isoforms of DAF-16 contribute unequally to longevity, metabolism, stress response, and development(Kwon *et al.*, 2010). Precisely how DAF-16 is able to discriminate between different signals and correctly couple each upstream signal with a downstream interactor to mount the proper transcriptional response is still not well understood.

1.2.1 Cytoplasmic regulators of DAF-16

The Insulin/Insulin-like Growth (IGF) Factor signaling (IIS) cascade is one of the best characterized upstream regulators of DAF-16 activity. The IIS pathway is extremely well

conserved and modulates longevity in worms, flies, and mammals (Tatar, 2003). Inactivation of the insulin-like receptor, or its downstream kinases, robustly extends *C.elegans* lifespan and increases stress resistance through *daf-16* (Kenyon *et al.*, 1993; Dorman *et al.*, 1995; Tissenbaum and Ruvkun, 1998; Hertweck *et al.*, 2004). The IIS is comprised of the insulin/IGF-1 receptor ortholog *daf-2* which, analogous to its mammalian counterparts, initiates a conserved PI-3 kinase cascade activating downstream kinases AGE-1/AAP-1, PDK-1, AKT-1, AKT-2, and SGK-1(Morris *et al.*, 1996; Paradis and Ruvkun, 1998; Paradis *et al.*, 1999; Hertweck *et al.*, 2004). AKT kinases in turn phosphorylate DAF-16 causing cytoplasmic sequestration and hence inhibition of DAF-16 (Figure 2). Perturbation of the IIS through mutation of its components leads to dephosporylation and nuclear translocation of DAF-16 (Henderson and Johnson, 2001; Lee *et al.*, 2001). Stimulated DAF-16 subsequently mounts a transcriptional response by activating or repressing a large number of downstream target genes ultimately extending lifespan and heightening stress resistance (Honda and Honda, 2002; Lee *et al.*, 2003; McElwee *et al.*, 2003; Murphy *et al.*, 2003; Halaschek-Wiener *et al.*, 2005). Interestingly, TGF-β dauer pathway has been proposed to converge onto the IIS pathway to regulate DAF-16 activity, thus modulate longevity (Shaw *et al.*, 2007). The regulation of DAF-16/FOXO activity by insulin signaling is paralleled in mammals, albeit in some cases more complex and less well-understood.

Another kinase cascade, Jun N-terminal Kinase 1 (JNK) signaling, also converges on DAF-16/FOXO to influence longevity. JNK is an evolutionarily conserved stress-responsive, mitogen-activated protein kinase (MAPK) signaling cascade primarily activated by internal and external stressors such as inflammatory cytokines, increased reactive oxygen species, and DNA damage (Karpac and Jasper, 2009). In addition, signal transduction through the JNK pathway regulates various biological processes such as apoptosis, cell survival, tumorigenesis, and embryonic development (Davis, 2000). Activation of JNK signaling has recently been shown to improve stress tolerance and extend lifespan in *C. elegans* and *Drosophila*. Flies

carrying a JNK phosphatase mutation or overexpressing the JNK kinase Hemipterous (JNKK/Hep) exhibit increased resistance to oxidative stress-inducing agents and live longer than their wild-type counterparts (Wang *et al.*, 2003; Libert *et al.*, 2008). Similarly, JNK cascade components are implicated in aging and stress response processes in the worm. Inactivating the JNK homolog *jnk-1* or JNK kinase *jkk-1* shortens lifespan and leads to hypersensitivity to oxidative and heat stress, whereas overexpressing *jnk-1* extends lifespan and improves stress resistance (Oh *et al.*, 2005). The effects of JNK cascade on stress tolerance and lifespan are mediated by DAF-16/FOXO in both flies and worms. In both organisms, JNK activation results in nuclear translocation of DAF-16/FOXO (Oh *et al.*, 2005; Wang *et al.*, 2005). In worms, it is established that upon stress JNK-1 directly phosphorylates DAF-16 on residues distinct from AKT phosphorylation sites promoting accumulation of DAF-16 in the nucleus (Oh *et al.*, 2005). Mounting evidence suggests that JNK and IIS pathways engage in cross-talk to coordinate regulation of DAF-16/FOXO (Wang *et al.*, 2005; Neumann-Haefelin *et al.*, 2008).

DAF-16/FOXO is also a target of the evolutionarily conserved MST family of kinases (Lehtinen *et al.*, 2006). The Mammalian Ste-20 like kinases (MST) and their *Saccharomyces cerevisiae* and *Drosophila* homologs are involved in mediating oxidative stress-induced cellular responses such as apoptosis (Harvey *et al.*, 2003; Ahn *et al.*, 2005; Lehtinen *et al.*, 2006). Upon activation by increased cellular oxidative stress, mammalian MST-1 directly phosphorylates FOXO3, thereby promoting FOXO's translocation into the nucleus and inducing neuronal cell death. *C. elegans* MST-1 ortholog CST-1 regulates DAF-16 in a conserved mechanism, through phosphorylation. While *cst-1* mutants display a relatively short lifespan, overexpressing *cst-1* promotes longevity through *daf-16.* The influence of *cst-1* on lifespan appears to occur independently of IIS (Lehtinen *et al.*, 2006). The interplay between the kinase cascades upstream of DAF-16 and how DAF-16 is able to accommodate inputs from these pathways needs to be further investigated.

In *C. elegans*, removal of the germline through ablation of germline precursor cells or mutation of the *glp-1* gene robustly extends lifespan (Hsin and Kenyon, 1999; Arantes-Oliveira *et al.*, 2002). The lifespan extension conferred by the inhibition of germline proliferation requires the activity of a nuclear hormone receptor *daf-12* as well as *daf-16* (Hsin and Kenyon, 1999). Both *kri-1*, which encodes an ankyrin repeat protein, and the *daf-9/daf-12* lipophilic-hormone signaling pathway transduce the reproductive signals from the germline to the intestine where they promote nuclear accumulation of DAF-16 in germline-defective animals (Lin *et al.*, 2001; Berman and Kenyon, 2006). Genetic epistasis analyses between germline ablated worms and *daf-2* mutants revealed that the IIS and lipophilic hormone signaling pathways extend lifespan synergistically, suggesting that they operate independently to regulate DAF-16 (Hsin and Kenyon, 1999) (Figure 2).

Evolutionarily conserved 14-3-3 proteins are small acidic proteins without a known enzymatic function. 14-3-3 proteins bind phosphoserine/threonine residues on a diverse array of target proteins (Durocher *et al.*, 2000). Due to interactions with innumerable proteins, 14-3-3 proteins are involved in major cellular processes such as cell cycle, signaling and stress response, DNA damage response, apoptosis (Tzivion *et al.*, 2001). 14-3-3 proteins mainly function as scaffolding molecules to regulate the activities of their partners. Interestingly, 14-3-3 factors can take on activator or repressor roles depending on their interactors and/or the cellular context (Tzivion *et al.*, 2001). 14-3-3 proteins employ multiple different mechanisms to regulate their target proteins: they prevent or promote association of their targets with other regulators, alter nuclear localization and change the intrinsic catalytic activity by modifying the conformation of their partners (Tzivion *et al.*, 2001). 14-3-3 proteins interact with and regulate the activity of DAF-16/FOXO transcription factors. 14-3-3 can recognize and bind to the phosphorylated residues on DAF-16/FOXO created by AKT phosphorylation (Cahill *et al.*, 2001; Brunet *et al.*, 2002). Once bound to DAF-16/FOXO in the nucleus, 14-3-3 proteins disrupt the association with DNA and facilitate nuclear export of DAF-16/FOXO proteins

(Cahill *et al.*, 2001; Obsil *et al.*, 2003; Berdichevsky *et al.*, 2006; Boura *et al.*, 2007; Li *et al.*, 2007). Interestingly, *C. elegans* 14-3-3 proteins exhibit dual functions in DAF-16 regulation, where they promote DAF-16's activation through scaffolding its association with a coactivator SIR-2.1 (Berdichevsky *et al.*, 2006; Wang *et al.*, 2006). It is yet unclear whether 14-3-3 homologs in mammals also activate FOXOs under specific conditions.

1.2.2 Nuclear cofactors of DAF-16

About a decade ago, an interesting observation suggested that the translocation of DAF-16 into the nucleus, although necessary for its activation, is not sufficient to stimulate its transcriptional activity (Lin *et al.*, 2001). Overexpressing a constitutively nuclear version of DAF-16, mutated for four AKT-phosphorylation sites (DAF-16a^{AM}::GFP), fails to induce dauer formation and extend lifespan (Lin *et al.*, 2001). In addition, high levels of wild-type DAF-16 can only confer a mild lifespan extension (Henderson and Johnson, 2001). Taken together, these observations indicate that DAF-16 activity must be governed inside the nucleus by other mechanisms and factors which ensure transcriptional specificity of DAF-16. Indeed, a growing body of evidence suggests that association with additional nuclear co-regulators is also necessary for nuclear DAF-16 activity (Essers *et al.*, 2005; Berdichevsky *et al.*, 2006; Berman and Kenyon, 2006; Lehtinen *et al.*, 2006; Wolff *et al.*, 2006; Li *et al.*, 2008).

1.2.2.1 SIR-2.1/14-3-3

SIR-2.1 is the *C. elegans* homolog of the budding yeast Nicotinamide Adenine Dinucleotide (NAD+)-dependent protein deacetylase Sir2p. *SIR2* belongs to a family of Silent Information Regulator (*SIR*) genes, collectively called sirtuins, that were originally identified as components of the transcriptional silencing machinery at the silent mating type loci in yeast (Ivy *et al.*, 1986; Rine and Herskowitz, 1987). Besides their established role in

transcriptional silencing, a growing body of evidence suggests that Sir2 and its orthologs across multiple species are important modulators of aging and age-associated diseases (Donmez and Guarente, 2010). In yeast, *sir-2*Δ mutation robustly shortens and overexpression increases lifespan (Kaeberlein *et al.*, 1999). Sir2p achieves this lifespan extension by suppressing recombination at the ribosomal DNA(rDNA) loci and minimizing the generation of rDNA circles which are detrimental to the organism (Kaeberlein *et al.*, 1999). Although it was initially proposed that the lifespan extension conferred by Caloric Restriction (CR) in yeast is also mediated by Sir2p (Lin *et al.*, 2000), later conflicting results have called this observation into question (Kaeberlein *et al.*, 2004; Kaeberlein and Powers, 2007). In flies, an analogous lifespan extension by overexpressing or overactivating dSir2 has been demonstrated (Rogina and Helfand, 2004; Wood *et al.*, 2004). Genetically introducing extra copies of the *dSir2* gene either in the whole animal or only in the neurons leads to a significantly longer lifespan in both male and female flies. As in yeast, mutation of *dSir2* abolished the longevity effects of CR, pointing to the conservation of Sir2 function in mediating the beneficial consequences of CR (Rogina and Helfand, 2004). Furthermore, administering Sir2-activating chemicals, such as resveratrol, increases lifespan in a Sir2-dependent manner in *Drosophila.* In agreement with the proposal that dSir2 is involved in CR-mediated lifespan extension (Rogina and Helfand, 2004), chemically-induced Sir2 activation did not synergize with CR to further increase lifespan (Wood *et al.*, 2004). As in yeast, and flies, overexpressing *sir-2.1* confers lifespan extension in *C. elegans* (Tissenbaum and Guarente, 2001; Viswanathan *et al.*, 2005; Berdichevsky *et al.*, 2006). Whereas mutation of *sir-2.1* shortens lifespan and heightens sensitivity to oxidative, heat, and DNA-damage induced stresses, overexpression of SIR-2.1 extends lifespan and improves stress resistance (Tissenbaum and Guarente, 2001; Berdichevsky *et al.*, 2006; Wang and Tissenbaum, 2006). Although *sir-2.1* has been initially implicated in the response to CR (Wood *et al.*, 2004; Wang and Tissenbaum, 2006), conflicting reports have made it difficult to establish a role for SIR-

2.1 in *C. elegans* CR pathways (Bass *et al.*, 2007; Greer and Brunet, 2009). A critical role for mammalian Sir2 (SIRT1) in the regulation of various CR-associated effects is beginning to emerge (Chen and Guarente, 2007; Donmez and Guarente, 2010). Increase in physical and metabolic activity as well as the long-lifespan conferred by reduced caloric intake is attenuated in SIRT1 knockout mice (Chen *et al.*, 2005; Boily *et al.*, 2008). Transgenic mice overexpressing SIRT1 (Bordone *et al.*, 2007; Banks *et al.*, 2008; Pfluger *et al.*, 2008) or mice with chemically-induced overactivation of SIRT1 (Barger *et al.*, 2008; Smith *et al.*, 2009) exhibit phenotypes of CR under *ad libitum* feeding conditions. Apart from its involvement in the CR effect, SIRT1 is shown to be important for DNA damage repair to promote genomic stability, protect mice from DNA damage-induced cancer formation, and regulate transcriptional changes during aging (Oberdoerffer *et al.*, 2008). Although through seemingly diverse mechanisms in different species, Sir2 has an evolutionarily conserved role in longevity determination.

In *C. elegans*, SIR-2.1 is thought to act as a cofactor to activate DAF-16 in conferring longevity as well as stress resistance (Tissenbaum and Guarente, 2001; Berdichevsky *et al.*, 2006; Wang *et al.*, 2006). Impairing *daf-16* activity in the long-lived *sir-2.1* overexpression strains leads to a significant reduction in lifespan (Tissenbaum and Guarente, 2001; Berdichevsky *et al.*, 2006). Besides lifespan extension, overexpresion of *sir-2.1* leads to increased heat and oxidative stress resistance and elevated expression of a direct DAF-16-target gene superoxide dismutase 3 (*sod-3)* in a *14-3-3*-dependent manner (Berdichevsky *et al.*, 2006). SIR-2.1 physically associates with DAF-16 and both members of the 14-3-3 proteins, PAR-5 and FTT-2 (Berdichevsky *et al.*, 2006; Wang *et al.*, 2006). Interactions between SIR-2.1 and DAF-16 are particularly improved upon heat stress and 14-3-3 proteins are required for this association to occur (Berdichevsky *et al.*, 2006). It is proposed then that upon stress, SIR-2.1 binds DAF-16 in the presence of 14-3-3 proteins and this complex promotes the activation of DAF-16 at its target genes (Berdichevsky *et al.*, 2006).

Interestingly, contrary to their roles in ensuring cytoplasmic retention of DAF-16 (Cahill *et al.*, 2001; Berdichevsky *et al.*, 2006; Li *et al.*, 2007), 14-3-3 proteins appear to positively regulate the transactivation of DAF-16 in the nucleus in the context of stress and SIR-2.1 activation (Berdichevsky *et al.*, 2006), emphasizing the multifaceted functions of 14-3-3 proteins on DAF-16/FOXO regulation. Initial studies investigating the link between *sir-2.1* and the insulin signaling pathway (IIS) implied that *sir-2.1* participated in IIS since combination of *sir-2.1* overexpression with *daf-2* mutation did not further increase lifespan (Tissenbaum and Guarente, 2001). However, subsequent demonstration that *sir-2.1* mutation did not suppress *daf-2* mutant longevity implicated *sir-2.1* to likely act independent of or upstream of IIS (Berdichevsky *et al.*, 2006). Due to these conflicting reports, it is unclear whether *sir-2.1* interacts with the insulin signaling pathway or whether it converges onto DAF-16 independent of insulin signaling.

In mammals, SIRT1 associates with and directly deacetylates FOXO proteins in a stress-dependent manner (Brunet *et al.*, 2004; Daitoku *et al.*, 2004; Motta *et al.*, 2004; Yang *et al.*, 2005). There are four FOXO family members in mammals: FOXO1, FOXO3a, FOXO4 and FOXO6 (Giannakou and Partridge, 2004). FOXO proteins are involved in many diverse biological processes including apoptosis, cell cycle regulation, differentiation, metabolism, immunity, stress response and development (Huang and Tindall, 2007; van der Horst and Burgering, 2007; Arden, 2008). In response to oxidative stress, such as hydrogen peroxide treatment, heat stress, or UV stress, FOXO1, FOXO3a and FOXO4 associate with acetyltransferases p300 and CREB-binding protein (CBP) and in turn become acetylated. Subsequently, SIRT1 binds and deacetylates FOXOs (Brunet *et al.*, 2004; Daitoku *et al.*, 2004; Motta *et al.*, 2004; van der Horst *et al.*, 2004). Interestingly, the consequences of SIRT1 deacetylation on FOXO activity are multifaceted. Depending on the cell line or conditions, SIRT1 can either activate or repress FOXOs' ability to induce their target genes (Giannakou and Partridge, 2004). Overall, under stressful conditions, SIRT1 inhibits the

apoptotic response by FOXO proteins and promotes FOXOs' ability to induce cell-cycle arrest and survival (Giannakou and Partridge, 2004). Whether the effects of SIRT1 in aging and age-related pathologies are mediated through FOXO transcription factors is yet to be determined.

1.2.2.2 SMK-1

C. elegans SMK-1 is a DAF-16 cofactor required for the longevity and stress response functions of DAF-16. SMK-1 is the *C. elegans* homolog of the soil-living amoeba *Dictyostelium* SMEK (suppressor of MEK null), originally identified to be involved in the chemotaxis response regulated by the MAP kinase pathway (Mendoza *et al.*, 2005). Loss of SMEK suppresses the chemotaxis and cell polarity defects and precocious gene expression caused by *erk1*$^{-}$ (extracellular singal-regulated kinase) and *mek1*$^{-}$ (ERK regulating kinase) mutations. SMEK also exhibits MEK1 independent functions such as cytokinesis and development (Mendoza *et al.*, 2005). SMEK proteins are highly conserved and are shown to be regulatory subunits of a PP4 protein phosphatase complex in *Drosophila,* yeast, and mammals (Gingras *et al.*, 2005; Chowdhury *et al.*, 2008). As part of the PP4 phosphatase complex, SMEK is implicated in a variety of cellular and physiological processes such as development (Spradling *et al.*, 1999), DNA damage repair (Wu *et al.*, 2004; Gingras *et al.*, 2005; Chowdhury *et al.*, 2008), apoptosis, (Mourtada-Maarabouni *et al.*, 2003), insulin-receptor substrate 4 expression (Mihindukulasuriya *et al.*, 2004), cell cycle regulation (Kittler *et al.*, 2004), asymmetric division of neuroblasts (Sousa-Nunes *et al.*, 2009), hepatic gluconeogenesis (Yoon *et al.*, 2010), transcriptional regulation of embryonic stem cell maintenance through Wnt-signaling (Lyu *et al.*, 2011), and aging (Wolff *et al.*, 2006).

SMK-1 is a specific modulator of DAF-16's transcriptional activity in conferring a longer lifespan and heightened stress response. SMK-1 is found to be expressed throughout development, predominantly residing in the nuclei of intestinal, neuronal, and hypodermal

cells, which coincide with the primary sites of action of active DAF-16 (Wolff *et al.*, 2006). Interestingly, among many phenotypes exhibited by insulin signaling mutants, all of which are mediated by DAF-16, *smk-1* is required for only a subset. Attenuating *smk-1* function can suppress the long lifespans as well as heightened immune, UV, and oxidative stress responses of *daf-2* mutants. However, *smk-1* is dispensable for thermotolerance, increased dauer formation, and delayed reproductive timing of *daf-2* mutant worms. SMK-1 regulates the activity of DAF-16 at a select subset of target genes thus ensuring functional specificity for DAF-16 (Wolff *et al.*, 2006). The molecular mechanism by which SMK-1 promotes the transactivation of DAF-16 is poorly understood. Exploring whether SMK-1 is part of a conserved phosphatase complex as it is in other organisms and whether this phosphatase activity may be important for the interactions between SMK-1, DAF-16 and additional regulatory players will provide insights into the molecular details of SMK-1 function in longevity.

1.2.2.3 BAR-1

bar-1, the *C. elegans* ortholog of β-catenin, is a regulator of dauer development, longevity, and oxidative stress response functions of DAF-16 (Essers *et al.*, 2005). *bar-1* is part of the evolutionarily conserved canonical Wnt-signaling cascade and through regulating *hox* gene expression, it carries out critical functions in *C. elegans* development such as migration of neuroblast cells, vulval, hypodermal, hermaphrodite seam cell, and male tail cell fate specification (Eisenmann, 2005). Apart from its functions in early development, *bar-1* is involved in dauer formation and longevity (Essers *et al.*, 2005). In worms, absence of *bar-1* prevents *daf-16*-dependent dauer formation promoted by loss of function mutations in *daf-2* receptor. Overexpression of *bar-1* enhances dauer entry thereby demonstrating that *bar-1* mediates the dauer induction by DAF-16 downstream of IIS. *bar-1* is also implicated in longevity determination since lack of *bar-1* activity substantically reduces wild-type lifespan,

similar to the effect of *daf-16* loss of function mutations (Essers *et al.*, 2005). In line with the observations that many longevity factors are involved in stress response regulation, *bar-1* plays a role in oxidative stress response. Similar to *daf-16* mutants, *bar-1* mutant worms display hypersensitivity to an oxidative-stress inducing agent paraquat whereas overexpressing *bar-1* confers resistance. BAR-1 likely counteracts the harmful effects of increased oxidative insults by promoting the transactivation of DAF-16 target genes such as superoxide dismutase 3 (*sod-3*) (Essers *et al.*, 2005). Interestingly, other components of the canonical Wnt signaling pathway, such as the *bar-1* inhibitor *pry-1* or transcription factor *pop-1* which acts downstream of *bar-1* (Eisenmann, 2005), are not required for the paraquat-induced elevation in *sod-3* expression, highlighting the specificity of *bar-1* in responding to oxidative stress (Essers *et al.*, 2005). BAR-1 is shown to physically associate with DAF-16 particularly under increased oxidative stress conditions (Essers *et al.*, 2005). In recent studies, *bar-1* is shown to be involved in protecting muscle cell integrity in a *C. elegans* model of muscular dystrophy through interactions with *daf-16* (Pasco *et al.*, 2010). However, the exact consequence of this interaction and the mechanism by which BAR-1 can promote DAF-16 activation is not yet elucidated.

The mammalian β-catenin ortholog also regulates FOXO transcription factor activity. Simultaneous overexpression or stabilization of β-catenin with overexpressed FOXO4 enhances the expression of FOXO target genes. Conversely, depleting endogenous β-catenin by siRNA treatment blunts the transcriptional response by overexpressed or overactivated FOXO in cells (Essers *et al.*, 2005). Thus, β-catenin facilitates FOXO's transcriptional activation in mammals. Moreover, FOXOs and β-catenin physically interact especially after cells are challenged by oxidative stress (Essers *et al.*, 2005). Taken together, these observations assign a substantial role to BAR-1/ β-catenin in modulating DAF-16/FOXO activity and contributing to aging and stress responses.

1.2.2.4 HSF-1

Heat shock factor 1 (*hsf-1*) in *C. elegans* is the ortholog of the stress responsive transcription factor HSF. By mediating the transactivation of molecular chaperones such as heat shock proteins (HSPs) and proteases upon acute cellular insults, HSFs mediate a prompt response to proteotoxic stress. Besides their vital roles as cellular stress sensors, HSF proteins also play key roles in developmental processes. In *D. melanogaster, Hsf* regulates oogenesis and larval development (Jedlicka *et al.*, 1997). In mice, HSF proteins are essential for many different aspects of development including early embryogenesis, brain and central nervous system development, as well as oogenesis and spermatogenesis (Akerfelt *et al.*, 2010). Several lines of evidence establish molecular chaperones and HSF proteins to be conserved longevity modulators. Overexpressing chaperones such as Hsp70 extends lifespan in *Drosophila* and *C. elegans* (Tatar *et al.*, 1997; Yokoyama *et al.*, 2002; Walker and Lithgow, 2003). Additional copies of *hsf1* in the fission yeast *Saccharomyces pombe* lengthens chronological lifespan (Ohtsuka *et al.*, 2011). Expression of HSPs are altered in long-lived dwarf mice (Swindell *et al.*, 2009). The contribution of HSFs to the maintenance of proteostasis is postulated to be at least part of the mechanism by which HSF proteins influence longevity (Morimoto, 2008).

hsf-1's role as a major longevity determinant in worms is established by observations that disruption of *hsf-1* function accelerates the decline of tissue integrity and aging, whereas overexpressing it prolongs lifespan and promotes stress resistance (Garigan *et al.*, 2002; Hsu *et al.*, 2003). Inactivating *hsf-1* suppresses the long-lifespan, pathogen resistance, and increased dauer formation of several IIS mutants (Hsu *et al.*, 2003; Morley and Morimoto, 2004; Singh and Aballay, 2006). Overexpressing *hsf-1* extends lifespan in a *daf-16*-dependent manner and depleting *hsf-1* in *daf-16* mutants does not further shorten lifespan, thereby suggesting that these factors work in a common pathway to influence longevity (Hsu *et al.*, 2003; Morley and Morimoto, 2004). Interestingly, while both DAF-16 and HSF-1 can

activate the transcription of independent sets of target genes, they cooperatively turn on the expression of a subset of genes under heat shock or reduced IIS conditions (Hsu *et al.*, 2003). Of note are a group of small heat shock proteins (sHSPs) including *hsp-16.1, hsp-16.49,* and *hsp-12.6* that are among the DAF-16/HSF-1 co-regulated genes (Hsu *et al.*, 2003). Among many other phenotypes, IIS mutants also exhibit delayed accumulation of protein aggregates which are exacerbated with age (Morley *et al.*, 2002). Inactivating *daf-16, hsf-1,* or sHSPs whose expression depend on *daf-16* and *hsf-1,* accelerates the accumulation of harmful protein aggregates in worms (Hsu *et al.*, 2003). It is thus postulated that molecular chaperone networks, HSF-1, and DAF-16 collaborate to mediate protection against the adverse effects of misfolded, aggregated proteins (Hsu *et al.*, 2003; Morimoto, 2008). These findings emphasize once again the prevalence and importance of transcriptional specificity that DAF-16 has to accomplish in order to properly respond to different stimuli.

1.2.2.5 TCER-1

C. elegans tcer-1 is a downstream effector of the germline proliferation pathway (Ghazi *et al.*, 2009). *tcer-1* is the ortholog of mammalian transcription elongation regulator *TCERG1*. In mammals, aside from its roles in transcription elongation, TCERG1 is beginning to emerge as a transcriptional regulator of versatile transcription factors. TCER1 acts as a coactivator of DACH1 (Dachshund homolog 1), a transcriptional regulator of retinal cell fate determination (Zhou *et al.*, 2010), and as a corepressor of CCAAT enhancer binding protein α (C/EBPα), a master regulator of hematopoetic differentiation (Koschmieder *et al.*, 2009; Moazed *et al.*, 2011).

When the active proliferation of germline stem cells is compromised either by genetic mutations or laser ablation of germline precursor cells, a signal is transduced from the gonad to the intestinal cells to promote DAF-16's translocation into the nucleus and initiate a transcriptional response that ultimately extends lifespan (Hsin and Kenyon, 1999; Lin *et al.*,

2001). Reduction of *tcer-1* function suppresses the long lifespan produced by germline ablation. On the other hand, *tcer-1* loss of function does not affect the longevity of either wild-type or long-lived *daf-2* mutant worms, thus *tcer-1* is a specific regulator of lifespan under conditions of attenuated germline proliferation (Ghazi *et al.*, 2009). Germline loss increases *tcer-1* expression in the intestine, which is required for the expression of a subset of DAF-16 target genes induced upon germline removal. Furthermore, overexpression of *tcer-1* is sufficient to prolong lifespan in wild type worms in a *daf-16* dependent manner. Interestingly, the effect of TCER-1 on DAF-16 activity is confined to the nucleus since it does not regulate the subcellular localization of DAF-16 (Ghazi *et al.*, 2009). Overall, *tcer-1* represents yet another nuclear DAF-16 coactivator, which can selectively promote the activation of DAF-16 at a specific subset of downstream promoters only when germline proliferation is diminished, albeit through an unknown mechanism.

1.2.2.6 GATA factors

GATA transcription factors are widely conserved in many species. GATA factors are named after the WGATAR consensus DNA motif they bind in order to regulate the expression of a plethora of genes crucial for development and differentiation. In vertebrates, six GATA transcription factors, GATA1-6, exist and these factors are divided into two subgroups depending on their spatial and temporal expression (Viger *et al.*, 2008). GATA1/2/3 factors are critical for hematopoietic cell differentiation (Weiss and Orkin, 1995) and the morphological development of brain, spinal cord and inner ear in vertebrates (George *et al.*, 1994; Nardelli *et al.*, 1999; Lillevali *et al.*, 2004). On the other hand, GATA4/5/6 factors participate in early developmental processes and have prominent roles in the formation of endodermal and mesodermal tissues such as the heart, gut, and gonads (Viger *et al.*, 2008). GATA proteins frequently cooperate with various transcriptional partners and cofactors to achieve specificity in gene expression (Viger *et al.*, 2008). *C. elegans* encodes 14 GATA

transcription factors, some of which have been shown to affect endoderm development and differentiation to form the worm digestive tract (Stainier, 2002).

Recently, GATA4/5/6 homologs in *C. elegans* have been shown to direct the transcriptional changes that occur during aging (Budovskaya *et al.*, 2008). A study looking for gene expression changes during aging identified more than 1,000 genes which were up- or downregulated as the worms grew older (Budovskaya *et al.*, 2008). Interestingly, a highly enriched consensus GATA transcription factor-binding motif shared among the promoters of the age-regulated genes pointed to GATA factors as modulators of the age-associated transcriptional variations. Specifically, *elt-3, elt-5,* and *elt-6* are found to be the GATA factors which influence longevity (Budovskaya *et al.*, 2008). Inactivating *elt-3* curtails the long lifespan of *daf-2* mutants, whereas knockdown of *elt-5* or *elt-6* extend lifespan in an *elt-3*-dependent fashion. Therefore, ELT-3 activation can promote youthfulness, whereas ELT-5 and ELT-6 counteract ELT-3 through inhibiting its expression, thus accelerating aging. Given that both GATA factors and DAF-16 act downstream of IIS to influence lifespan and that there is a significant enrichment of the GATA-binding motif on the promoters of DAF-16 target genes, it is likely that this intriguing network of GATA factors interact with DAF-16 (Budovskaya *et al.*, 2008). The detailed mechanism by which DAF-16 and GATA factors cooperate to modulate aging remains to be investigated.

1.2.2.7 CTBP-1

C-terminal Binding Protein (CtBP) is a highly conserved NAD/NADH-dependent transcriptional repressor (Chinnadurai, 2002). The cellular NAD/NADH ratio impacts CtBP activity and interactions with transcription factors (Zhang *et al.*, 2002). Through recruiting chromatin modifying enzymes such as histone methyltransferases and demethylases, CtBP inhibits the transcriptional activation of promoter-bound transcription factors (Shi *et al.*, 2003). Known physiological functions of CtBP proteins include harmonizing transcription

factor activities during development (Hildebrand and Soriano, 2002), maintaining Golgi morphology, regulating neuronal synapse formation (Chinnadurai, 2003), and to enhancing tumorigenesis by antagonizing apoptosis, promoting malignancy and repressing tumor suppressors (Chinnadurai, 2009).

The *C. elegans* CtBP homolog CTBP-1 also possesses transcriptional corepressor functions as revealed by its ability to inhibit VP16-activated expression of a luciferase reporter in mammalian COS-1 cells (Nicholas *et al.*, 2008). CTBP-1 has been recently identified to be a DAF-16 corepressor (Chen *et al.*, 2009). Abrogating *ctbp-1* by mutation or RNAi extends lifespan in worms. The lifespan extension in *ctbp-1* mutants is completely dependent on active *daf-16* and does not further extend the long lifespan of *daf-2* mutants, thereby suggesting that *ctbp-1* acts downstream of IIS (Chen *et al.*, 2009). Genetic evidence also revealed that *ctbp-1* likely acts downstream of *sir-2.1* to modulate lifespan. As is the case for many recently discovered DAF-16 coregulators, CTBP-1 influences specific functions of DAF-16 in stress responses where *ctbp-1* inactivation only confers resistance to oxidative and heat stress but not DNA damage, starvation or pathogen stress (Chen *et al.*, 2009). Processes represented by genes whose expression is altered in *ctbp-1* mutants are comprised of lipid metabolism, stress response and cellular transport among others. Lipid metabolism plays an important part in the lifespan extension effects seen in *ctbp-1* mutant worms since the increased expression of a Triacylglyceride (TAG) lipase *lipl-7* contributes to the long lifespan of *ctbp-1* mutants possibly through reducing TAG levels in these worms (Chen *et al.*, 2009). Although an in-depth understanding of the mechanism whereby CTBP-1 regulates DAF-16 activity is lacking, it is likely that changing NAD/NADH levels in the cell may act as a signal to promote CTBP-1 action on DAF-16 particularly to regulate lipid metabolism and lifespan. The emerging roles of mammalian CtBP proteins in lipid regulation (Kajimura *et al.*, 2008; Nicholas *et al.*, 2008; Sue *et al.*, 2008) raise an interesting possibility that CtBP may play analogous roles in regulating mammalian FOXO transcription factor

activity and aging.

1.2.2.8 ETS-4

ETS-4 transcription factor was recently established as a transcriptional modulator of longevity and identified as a potential DAF-16 corepressor (Thyagarajan *et al.*, 2010). *C. elegans ets-4* belongs to a family of metazoan ETS (E28 Transformation-specific Sequence) transcription factors and is the homolog of mammalian SAM pointed domain containing ETS transcription factor (*SPDEF)* (Thyagarajan *et al.*, 2010). ETS factors share an ETS DNA binding motif that has affinity to a GGAA/T core recognition sequence (Nye *et al.*, 1992; Graves and Petersen, 1998). ETS transcription factors regulate the expression of genes involved in many biological processes including development, cellular differentiation, proliferation, apoptosis and are particularly important for cancer biology in various tissues because of their involvement in tumorigenesis and angiogenesis (Maroulakou and Bowe, 2000; Sementchenko and Watson, 2000; Seth and Watson, 2005; Jedlicka and Gutierrez-Hartmann, 2008). The SPDEF transcription factor is implicated in several disease phenotypes in humans and mice. *SPDEF* expression levels are altered in breast and prostate cancers leading to aberrant gene expression in the tumors (Feldman *et al.*, 2003; Gu *et al.*, 2007). *SPDEF* also plays a role in metastasis by mounting transcriptional responses to affect cellular migration and invasion in various tumors (Oettgen *et al.*, 2000; Chen *et al.*, 2002; Gunawardane *et al.*, 2005; Turner *et al.*, 2007) and participates in epithelial cell differentiation pathways (Gregorieff *et al.*, 2009). Several different mechanisms of regulation exist to govern ETS factor activity: ETS proteins can cooperate with additional transcription factors to activate or repress transcription, gain specificity through homo/heterodimerization, be regulated by posttranslational modifications as well as spatial and temporal expression (Hart *et al.*, 2000).

Ten ETS homologs exist in *C. elegans.* Emerging evidence from worm studies points

to a conserved role of ETS factors in development: *lin-1,* the first ETS factor characterized in worms, acts as a downstream effector of MAP kinase signaling and has key roles in vulval, male tail, excretory system, and ectoderm development (Ferguson and Horvitz, 1985; Han and Sternberg, 1990; Beitel *et al.*, 1995; Hart *et al.*, 2000). Another ETS ortholog *ast-1* is essential for neuronal differentiation and pharyngeal morphogenesis (Schmid *et al.*, 2006). So far, only *ets-4* has been implicated in longevity determination. Inactivating *ets-4* significantly prolongs lifespan in worms. Although *ets-4* mutation also causes a delay in larval development and reduction in brood size, RNA interference (RNAi) experiments allowed uncoupling of the developmental and longevity phenotypes of *ets-4* (Thyagarajan *et al.*, 2010). In order to influence lifespan, ETS-4 specifically functions in the intestine, the major site of action for DAF-16 to modulate lifespan. ETS-4 regulates the expression of a number of intestinally-expressed genes that are involved in lipid/fatty acid metabolism and aging (Thyagarajan *et al.*, 2010). Moreover, expression of a significant proportion of ETS-4 regulated genes is altered throughout aging thus further supporting a role for ETS-4 as a transcriptional contributor to longevity. The observations that the lifespan extension exhibited by *ets-4* mutants is fully dependent on *daf-16* and that ETS-4-regulated genes significantly overlap with a subset of DAF-16-regulated genes suggest that *ets-4* engages *daf-16* to regulate the expression of aging-genes and modulate lifespan (Thyagarajan *et al.*, 2010). Unlike its prominent longevity phenotypes, the *ets-4* mutant does not display any dauer, heat, or oxidative stress phenotypes. This intriguing observation delineates ETS-4 as a specific repressor of DAF-16 in lifespan modulation. Whether ETS-4 physically associates with DAF-16, how it specifically regulates DAF-16 activity at only a subset of target genes, whether it cooperates with additional interactors, and if the mammalian ETS-4 ortholog *SPDEF* similarly affects FOXO activities are questions that remain to be answered.

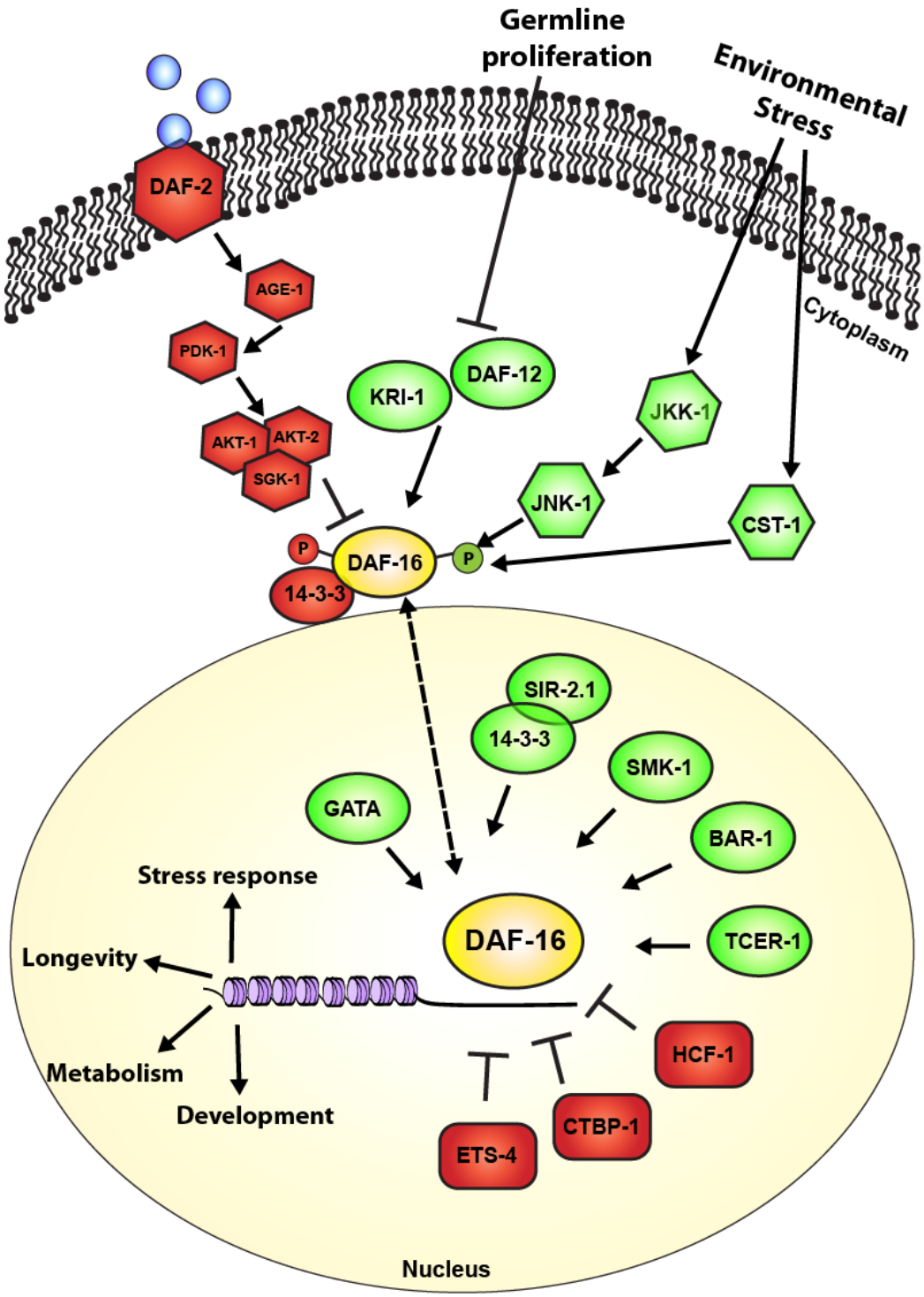


Figure1.2. Regulation of the DAF-16/FOXO longevity determinant.

1.3 SKN-1 longevity and stress response factor (Figure 1.3)

Skinhead Family Member 1 (SKN-1) is the only *C. elegans* ortholog of mammalian NF-E2-related factors (Nrf1, 2, and 3) and *Drosophila* CncC which are central mediators of acute and chronic defenses against oxidant-induced cellular stress (An and Blackwell, 2003; Nguyen *et al.*, 2003; Kobayashi and Yamamoto, 2006; Sykiotis and Bohmann, 2008; Sykiotis and Bohmann, 2010). Nrf transcription factors have long been established to be deployed in the face of oxidative or xenobiotic stress to mount a transcriptional detoxification response (Sykiotis and Bohmann, 2010). Nrf proteins belong to a highly conserved class of Cap-n-Collar basic leucine zipper transcription factors (Mohler *et al.*, 1991; Bowerman *et al.*, 1992; Chan *et al.*, 1993; Moi *et al.*, 1994; Kobayashi *et al.*, 1999). Although the oxidative stress response roles of Nrf2 has been studied more extensively, Nrf1 and Nrf3 functions are beginning to emerge. Nrf2 being the most potent transcriptional activator, all three Nrfs are capable of inducing overlapping and distinct sets of antioxidant and detoxifying enzymes basally and under heightened oxidative insults (Sykiotis and Bohmann, 2010). Nrfs specifically recognize and bind an Antioxidant Response Element (ARE) at the promoters of their immediate target genes. Due to the action of regulatory repressors such as Keap1, Nrf2 mainly resides in the cytoplasm under non-stressful conditions where its activity is kept in check both at the level of subcellular localization and protein stability (Sykiotis and Bohmann, 2010). Coordinated action of multiple kinase cascades and regulatory pathways facilitate Nrf2 accumulation in the nucleus upon increased oxidative stress where it commences a Phase II detoxification response. Nrf isoforms play both overlapping and non-redundant roles during cellular stress response, development, and aging. Single knockout of *nrf1* results in mid-late embryonic lethality whereas knocking out *nrf2* or *nrf3* alone does not produce developmental phenotypes (Sykiotis and Bohmann, 2010). Interestingly, *nrf2*$^{-/-}$ mice are highly sensitive to environmental stress and develop age-dependent pathologies including neurodegeneration caused by myelin sheath destruction and an autoimmune disease

reminiscent of human Lupus Erythematosus (Sykiotis and Bohmann, 2010). Moreover, due to its roles in tumorigenesis, Nrf2 is beginning to emerge as a candidate for anti-cancer therapies (Sykiotis and Bohmann, 2008). How each of the three Nrf isoforms are deployed differentially and how their functions are coordinated is not fully understood. Although Nrfs are primarily expressed in overlapping tissues, they differ in terms of their subcellular localization as Nrf2 is mainly cytoplasmic under basal conditions yet Nrf1 and Nrf3 are membrane proteins occupying the ER. In addition, interactions with different subsets of regulatory partners as well as context-dependent regulation may underlie the divergent functions of Nrfs in mammals (Sykiotis and Bohmann, 2010).

The multifaceted functions of the *C. elegans* Nrf ortholog SKN-1 transcription factor comprise early embryonic development, orchestration of the cellular oxidative stress response, and aging (Bowerman *et al.*, 1992; An and Blackwell, 2003; Tullet *et al.*, 2008). A single *skn-1* locus produces three different protein isoforms, SKN-1A/B/C (www.wormbase.org). Unlike their mammalian counterparts, tissue specific expression of SKN-1 isoforms represents at least one mode of regulation of different SKN-1 functions in worms. The *skn-1* isoforms display varying tissue expression patterns where the B isoform is predominantly expressed in two sensory ASI neurons while the A and C isoforms are found in the pharynx and the intestine (An and Blackwell, 2003; Bishop and Guarente, 2007). SKN-1 plays vital roles from early development to adulthood. *skn-1* was first discovered in a screen looking for maternal effect lethal mutations lacking pharyngeal cells (Bowerman *et al.*, 1992). SKN-1protein is required for the specification of the EMS blastomere fate in 4-cell embryos through directly inducing the expression of *med-1* and *med-2* GATA factors thereby giving rise to the digestive tract of the animal (Bowerman *et al.*, 1992; Bowerman *et al.*, 1993; Maduro *et al.*, 2001).

Despite sharing similarities with the protein structure of Nrf factors such as the DNA binding and transactivation domains, SKN-1 lacks the bZIP dimerization domain essential for

the functionality of Nrfs. However, SKN-1 shares a striking functional homology with Nrf proteins as evidenced by its central role in mounting a cellular detoxification defense against oxidative stress (An and Blackwell, 2003). As is the case for its mammalian Nrf2 counterpart, SKN-1 in the intestine is mostly cytoplasmic during unstressed conditions. Upon exposure to chemicals that produce high levels of reactive-oxygen species (ROS) or cause cellular damage, SKN-1 translocates into the intestinal nuclei and initiates a robust transcriptional induction of Phase II detoxifying enzymes including γ-glutamine cysteine synthetase 1(*gcs-1*), glutathione S-transferases (*gst*), and UDP-flucuronosyl/glucosyl transferases (*ugt)* (An and Blackwell, 2003; Xu *et al.*, 2005; Oliveira *et al.*, 2009). Despite its minimal cytoplasm to nucleus shuttling during unstressed conditions, SKN-1 constitutively activates or represses the expression of a subset of target genes involved in detoxification and stress response, albeit at a low level (Oliveira *et al.*, 2009). Interestingly, the SKN-1-mediated transcriptional program is adaptable and varies based on the chemical nature of toxic insults. Exposure of worms to a heavy metal sodium arsenite creates a SKN-1-dependent transcriptional response that is distinct from that created by an organic peroxide (Oliveira *et al.*, 2009). This intriguing complexity in the activation of SKN-1 during oxidative stress points to diverse mechanisms at work that need to ensure proper regulation of SKN-1 activity.

Several upstream regulatory pathways participating in the regulation of SKN-1 have been identified. SKN-1 is subject to inhibitory phosphorylation by Glycogen Synthase Kinase-3 (GSK-3) which prevents nuclear accumulation of SKN-1 in the intestine under basal conditions thereby avoiding unnecessary induction of the Phase II detoxification response (An *et al.*, 2005). The repressive action of GSK-3 on SKN-1 is counteracted by the evolutionarily conserved stress responsive p38 MAP Kinase pathway. Upon elevated oxidative stress, SKN-1 is directly phosphorylated by PMK-1, the downstream effector of the p38 MAPK cascade, which relieves the inhibitory effects of GSK-3 and facilitates nuclear accumulation of SKN-1

and induction of Phase II gene expression (Inoue *et al.*, 2005). In addition, SKN-1 is subject to inhibitory phosphorylation by the AKT kinases in the IIS cascade, where it is retained in the cytoplasm under normal conditions. Reduction of IIS allows for increased nuclear translocation and target gene expression by SKN-1, contributing to elevated protection against oxidative stress (Tullet *et al.*, 2008). Besides subcellular localization, SKN-1 activity is further governed at the level of protein stability. Several core subunits of the proteasome complex such as the WD-repeat protein *wdr-23* and *cul-4/ddb-1* ubiquitin ligase promote ubiquitin-mediated degradation of SKN-1 transcripts under normal conditions (Kahn *et al.*, 2008; Choe *et al.*, 2009). It is postulated that exposure to oxidative stress leads to stabilization of SKN-1 through inhibiting the proteasome complex and allows for nuclear accumulation and activation of SKN-1 (Kahn *et al.*, 2008; Choe *et al.*, 2009). In mammals, GSK-3 is similarly involved in ARE gene regulation, p38 MAPK affects Nrf2 transcriptional activation, and Nrf proteins are subject to regulation by ubiquitin-mediated degradation, thereby reiterating the functional conservation between SKN-1 and the mammalian Nrf proteins (Sykiotis and Bohmann, 2010).

Another very important postembryonic function of SKN-1 is longevity determination. *skn-1* mutant worms are short-lived whereas overexpressing SKN-1 prolongs lifespan (An and Blackwell, 2003; Tullet *et al.*, 2008). The specific action of SKN-1 in ASI sensory neurons mediates the long lifespan of dietary restricted (DR) worms suggesting that SKN-1 is a sensor of the nutritional status in *C. elegans* (Bishop and Guarente, 2007). Recently, SKN-1 has been shown to be a downstream effector of IIS in longevity modulation (Tullet *et al.*, 2008). SKN-1 protein is directly phosphorylated by AKT kinases in IIS and is sequestered in the cytoplasm. When components of the IIS are mutated, SKN-1 freely enters the nucleus and contributes to longevity through transactivation of its target genes. In contrast to the DR condition, IIS regulation of SKN-1 is confined to the intestine pointing to an intriguing mechanism of differential regulation of SKN-1 based on different upstream stimuli (Tullet *et*

al., 2008). Whether SKN-1 and DAF-16 collaborate to contribute to extended lifespan downstream of IIS is still unclear (Tullet *et al.*, 2008). However, SKN-1 is postulated to work together with DAF-16 to lengthen lifespan under conditions of reduced translation initiation (Wang *et al.*, 2010). In an RNAi screen looking for inducers of SKN-1 target gene *gcs-1* expression, a set of translation initiation genes previously shown to be involved in lifespan determination were identified. Subsequent work established SKN-1 as a major mediator of the effects of translation inhibition on stress tolerance and longevity (Wang *et al.*, 2010). The longevity functions of SKN-1 is evolutionarily conserved since the *Drosophila* SKN-1 ortholog CncC also participates in determining the rate of aging (Sykiotis and Bohmann, 2008). Given the high degree of functional conservation between SKN-1 and Nrfs and the involvement of mammalian Nrf2 in the development of age-related diseases such as neurodegeneration, autoimmune dysfunction, and cancer (Sykiotis and Bohmann, 2010), it is highly plausible that Nrf proteins play key roles in the biology of aging in humans.

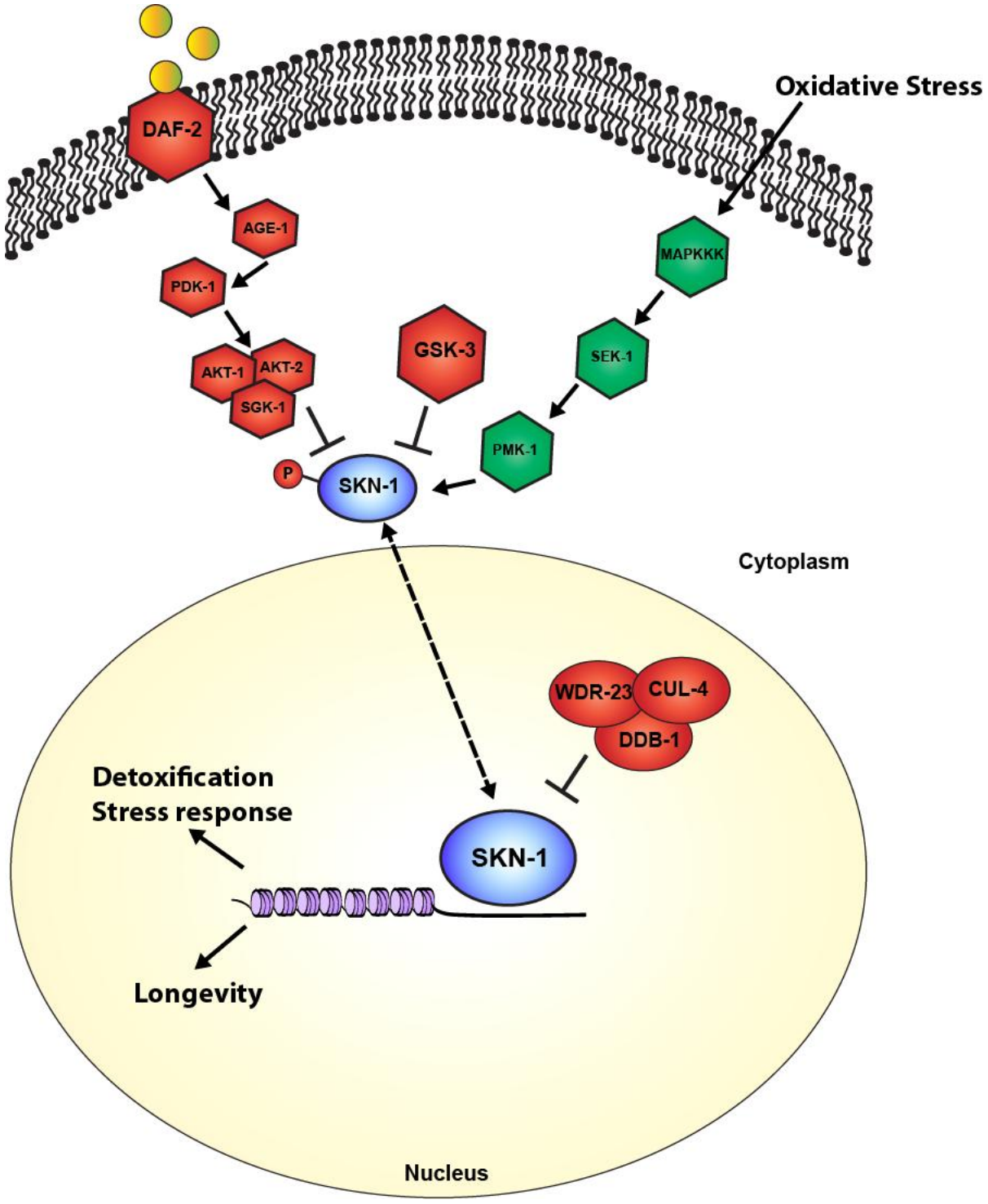


Figure 1.3. Regulation of the longevity and stress response modulator SKN-1.

CHAPTER 2

THE EVOLUTIONARILY CONSERVED LONGEVITY DETERMINANTS HCF-1 AND SIR-2.1 COLLABORATE TO REGULATE DAF-16[1]

ABSTRACT

The conserved DAF-16/FOXO transcription factors and SIR-2.1/SIRT1 deacetylases are critical for diverse biological processes, particularly longevity and stress response, and complex regulation of DAF-16/FOXO by SIR-2.1/SIRT1 is central to appropriate biological outcomes. *Caenorhabditis elegans* Host Cell Factor 1 (HCF-1) is a longevity determinant previously shown to act as a co-repressor of DAF-16. We report here that HCF-1 represents an integral player in the regulatory loop linking SIR-2.1 and DAF-16 in worms. Genetic analyses showed that *hcf-1* acts downstream of *sir-2.1* to influence lifespan and oxidative stress response in *C. elegans.* Gene expression profiling revealed a striking 80% overlap between the DAF-16 target genes responsive to *hcf-1* mutation and *sir-2.1* overexpression. Subsequent GO-term analyses of HCF-1 and SIR-2.1-coregulated DAF-16 targets suggested that HCF-1 and SIR-2.1 together regulate specific aspects of DAF-16-mediated transcription particularly important for aging and stress responses. Protein-protein association studies demonstrated that SIR-2.1 and HCF-1 form protein complexes. Our findings uncover a novel interaction between the key longevity determinants SIR-2.1/SIRT1 and HCF-1, and provide

[1] Chapter 2 is modified from Rizki G, Iwata TN, Li J, Riedel CG, Picard CL, Jan M, Murphy CT, and Lee SS, *PloS Genetics* (accepted). I, together with JL, CGR., CLP, MJ, CTM, and SSL conceived and performed the experiments and analyzed the data; I performed all the *C. elegans* experiments except co-IPs and *sir-2.1*(O/E) microarrays; JL performed the *C. elegans* co-IPs of HCF-1 and SIR-2.1, CGR performed the co-IP and mass spectrometry analyses with 14-3-3; M J and CTM performed the *sir-2.1*(O/E) microarrays; CLP assisted with microarray analyses.

new insights into the complex regulation of DAF-16/FOXO proteins.

INTRODUCTION

The Insulin/Insulin-like Growth Factor-1(IGF-1) signaling (IIS) cascade is one of the most highly conserved and best characterized longevity pathways in eukaryotes. When stimulated, the insulin/IGF-1 like receptors initiate a kinase cascade that leads to the phosphorylation, and cytoplasmic retention of the main downstream effectors, Forkhead box, Class O (FOXO) transcription factors. Reduction in IIS signaling leads to the dephosphorylation of FOXO, allowing nuclear translocation and transcriptional activation of FOXO (Burgering and Kops, 2002; Kenyon, 2010). The *C. elegans* FOXO ortholog DAF-16, as well as the *Drosophila,* mouse, and human FOXO transcription factors are all critical for longevity, metabolism, and stress response (Kenyon *et al.*, 1993; Lin *et al.*, 1997; Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004; Arden, 2008; Kappeler *et al.*, 2008; Wang *et al.*, 2008; Willcox *et al.*, 2008; Li *et al.*, 2009; Yuan *et al.*, 2009), suggesting that the mechanisms underlying FOXOs' ability to affect physiology are highly conserved across species. Indeed, much of our understanding of FOXO regulation comes from studies done on *C. elegans* DAF-16. When activated, DAF-16 selectively regulates the transcription of a large number of genes which cumulatively act to elevate stress resistance, alter metabolic and developmental responses, improve immunity, and extend lifespan (Lee *et al.*, 2003; McElwee *et al.*, 2003; Murphy *et al.*, 2003; Halaschek-Wiener *et al.*, 2005). To integrate many different environmental stimuli and coordinate proper transcriptional responses, DAF-16 activity must be tightly controlled. DAF-16 activity is known to be regulated by post-translational modifications, nuclear/cytoplasmic translocation and association with transcriptional co-regulators. Although necessary for its activation, translocation of DAF-16 into the nucleus is not sufficient to stimulate its transcriptional activity (Lin *et al.*, 2001). Association with additional co-factors is also necessary for nuclear DAF-16 activation (Essers *et al.*, 2005;

Berdichevsky *et al.*, 2006; Berman and Kenyon, 2006; Lehtinen *et al.*, 2006; Wolff *et al.*, 2006 ; Li *et al.*, 2008). Little is known about the interplay between DAF-16 and its nuclear regulators and how these multiple factors coordinately act on DAF-16 to ensure proper transcriptional outcomes.

SIR-2.1, the *C. elegans* homolog of the yeast NAD+-dependent protein deacetylase Sir2p, is an important DAF-16 co-factor. SIR-2.1 is thought to activate DAF-16 in conferring longevity as well as stress resistance (Tissenbaum and Guarente, 2001; Berdichevsky *et al.*, 2006; Wang *et al.*, 2006). Heat stress stimulates the physical association of SIR-2.1 with DAF-16 via the scaffolding proteins 14-3-3, which promote the transactivation of DAF-16 through an unknown mechanism (Berdichevsky *et al.*, 2006; Wang *et al.*, 2006). Overexpression of Sir2 homologs in worms, yeast and flies extends lifespan (Kaeberlein *et al.*, 1999; Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004; Berdichevsky *et al.*, 2006), emphasizing the evolutionarily conserved role of Sir2 in longevity determination. In mammals, SIRT1 associates with and directly deacetylates FOXO1, 3, and 4 in a stress-dependent manner (Brunet *et al.*, 2004; Daitoku *et al.*, 2004; Motta *et al.*, 2004; Yang *et al.*, 2005). However, the exact mechanism whereby SIR-2.1/SIRT1 affects DAF-16/FOXO activity and whether additional factors are involved in the regulation of DAF-16/FOXO by SIR-2.1/SIRT1 is not well understood.

Host Cell Factor-1 (HCF-1) belongs to a family of highly conserved HCF proteins and acts as a nuclear co-repressor of DAF-16 (Lee and Herr, 2001; Li *et al.*, 2008). Inactivating *hcf-1* robustly extends lifespan and confers oxidative stress resistance in a *daf-16*-dependent manner in *C. elegans*. In the nucleus, HCF-1 associates with DAF-16 and limits its access to a subset of target gene promoters (Li *et al.*, 2008). *C. elegans* HCF-1 shares high structural homology with two mammalian counterparts, HCF-1 and HCF-2 (Lee and Herr, 2001). Although mammalian HCF-1 has been studied extensively, HCF-2 functions remain largely unknown. Mammalian HCF-1 was originally identified as a binding partner of the Herpes

Simplex Virus VP16 transcription factor (Gerster and Roeder, 1988). Apart from VP16, HCF1 has been shown to associate with a number of transcription factors to stimulate or repress their transactivation properties (Lu *et al.*, 1997; Gunther *et al.*, 2000; Lu and Misra, 2000; Piluso *et al.*, 2002; Wysocka *et al.*, 2003; Tyagi *et al.*, 2007). HCF-1 is an important regulator of cellular proliferation as it promotes progression through multiple phases of the cell cycle via assembling transcriptional complexes to modulate E2F transcription factor activities (Julien and Herr, 2004; Tyagi *et al.*, 2007). Whether mammalian HCF proteins function as conserved FOXO regulators has yet to be determined.

In this study, we sought to examine whether the two conserved DAF-16/FOXO nuclear regulators, HCF-1 and SIR-2.1/SIRT1, functionally interact in worms. We found that *hcf-1* acts downstream of *sir-2.1* to regulate *daf-16* and thereby modulates lifespan and oxidative stress response in *C. elegans*. We showed that HCF-1 and SIR-2.1 regulate a common subset of DAF-16 target genes important for ensuring longevity and stress response. Our findings uncover a new regulatory mechanism between the critical longevity determinants DAF-16/FOXO and SIR-2.1/SIRT1, and implicate an important role of HCF-1 in aging and age-related diseases in diverse organisms.

RESULTS

C. elegans hcf-1 acts downstream of *sir-2.1* to modulate longevity and oxidative stress responses

In *C. elegans*, inactivation of *hcf-1* results in a robust lifespan extension, as well as improved survival upon exposure to oxidative stress, in a manner dependent on *daf-16.* In its role in longevity and stress response, HCF-1 inhibits DAF-16 activity by physically associating with DAF-16 and limiting DAF-16 localization to a subset of downstream target promoters (Li *et al.*, 2008). In the context of cell cycle progression, mammalian HCF-1 is known to regulate the activities of various transcription factors by promoting the formation of

transcriptional regulatory complexes (Wysocka *et al.*, 2003; Tyagi and Herr, 2009). We reasoned that HCF-1 in *C. elegans* may function similarly and, in conjunction with other transcriptional regulators, act to fine tune DAF-16 activity. As SIR-2.1 is a well-known, evolutionarily conserved longevity determinant that activates DAF-16 (Berdichevsky *et al.*, 2006), we explored whether HCF-1 and SIR-2.1 functionally interact to regulate DAF-16. As a first step, we examined the putative functional connection between *hcf-1* and *sir-2.1* in lifespan modulation by performing genetic analyses. We compared the lifespan of *hcf-1(pk924)* and *sir-2.1(ok434)* single mutants to that of *sir-2.1(ok434) hcf-1(pk924)* double mutants. Both *hcf-1* and *sir-2.1* alleles used in this analysis are putative null mutants (Wang and Tissenbaum, 2006; Li *et al.*, 2008). As previously described, *hcf-1(pk924)* mutant worms displayed a mean lifespan >20% longer than that of wild type and the *hcf-1(pk924)* long-lived phenotype was fully suppressed by *daf-16(mgDf47)* mutation (Figure 2.1A and (Li *et al.*, 2008)). *sir-2.1(ok434)* mutants exhibited lifespan always substantially shorter than that of *hcf-1(pk924)* (Figure 2.1A; Table 2.1A). We found that all four independent lines of the double mutants exhibited lifespans indistinguishable from that of *hcf-1(pk924)* single mutant worms, and significantly longer than that of *sir-2.1(ok434)* mutants (Figure 2.1A; Table 2.1A), suggesting that *sir-2.1* is not required for *hcf-1(pk924)* mutation to extend lifespan. Our genetic data suggest two possibilities: one is that *hcf-1* and *sir-2.1* may work independently and that *sir-2.1* inactivation does not affect *hcf-1(pk924)* mutant longevity. On the other hand, since the lifespan of the double mutant is similar to that of *hcf-1(pk924)* single mutant, *hcf-1* may act downstream of *sir-2.1*. To distinguish between these two possibilities, we examined the effect of overexpressing *sir-2.1* in worms harboring the *hcf-1* mutation. In *C. elegans*, overexpressing *sir-2.1* confers a lifespan extension phenotype that is dependent on *daf-16* (Tissenbaum and Guarente, 2001; Berdichevsky *et al.*, 2006). We reasoned that if *hcf-1* and *sir-2.1* work independently, then combining *hcf-1* inactivation with *sir-2.1* overexpression should further increase lifespan. By contrast, if *hcf-1* and *sir-2.1* work in the

same pathway, and *hcf-1* is genetically downstream of *sir-2.1*, then overexpression of *sir-2.1* should not cause further lifespan extension in *hcf-1(pk924)* mutants. To examine this, we utilized the long-lived, low-copy *sir-2.1* overexpressor strain NL3909 *pkIs1642 [unc-119 sir-2.1]* (*pkIs1642[sir-2.1*(O/E)*]*) (Viswanathan *et al.*, 2005; Berdichevsky *et al.*, 2006) to generate *hcf-1(pk924);pkIs1642[sir-2.1*(O/E)*]* strains. As a control, we outcrossed the *pkIs1642* strain and showed that it continues to extend lifespan compared to its transgenic control NL3908 *pkIs1641 [unc-119]* (*pkIs1641[sir-2.1*(wt)*]*) under our assaying conditions (Figure 2.3A; Table 2.1G). Furthermore, we knocked-down *sir-2.1* in the *pkIs1642* strain to show that the lifespan increase is indeed dependent on *sir-2.1* (Figures 2.3B-D; Table 2.1H). *hcf-1(pk924)* and *pkIs1642[sir-2.1*(O/E)*]* worms lived longer than N2 wild type or *pkIs1641[sir-2.1*(wt)*]* transgenic controls by 28% and 17%, respectively (Figure 2.1B, Tables 2.1B,G). Interestingly, the *hcf-1(pk924);pkIs1642[sir-2.1*(O/E)*]*) worms exhibited a lifespan very similar to, or in some cases shorter than, that of *hcf-1(pk924)* mutants (Figure 2.1B, Table 2.1B). However, in none of the *hcf-1(pk924);pkIs1642[sir-2.1*(O/E)*]*) isolates generated did we observe a lifespan longer than that of *hcf-1(pk924)* mutants (Table 2.1B). These data support the hypothesis that *hcf-1* acts in the same genetic pathway as *sir-2.1*. Considering our previous findings that *hcf-1* can robustly extend the lifespans of long-lived insulin signaling and germline proliferation mutants (Li *et al.*, 2008), our current observation that overexpression of *sir-2.1* cannot further enhance longevity in worms lacking *hcf-1* indicates that the genetic interaction between *hcf-1(-)* and *sir-2.1*(O/E) is specific.

In addition to their lifespan effects, both HCF-1 and SIR-2.1 regulate the ability of DAF-16 to respond to a variety of environmental stress cues. Adult *hcf-1(pk924)* mutant worms are resistant to oxidative- and heavy metal-stress (Li *et al.*, 2008). Likewise, *sir-2.1* overexpression is protective against exposure to oxidative as well as heat stress, while *sir-2.1* mutation increases sensitivity to oxidative, heat, and UV-induced environmental insults (Berdichevsky *et al.*, 2006; Wang and Tissenbaum, 2006). To further investigate the genetic

relationship between *hcf-1* and *sir-2.1*, we analyzed the response of *sir-2.1(ok434) hcf-1(pk924)* double mutants and *hcf-1(pk924);pkIs1642[sir-2.1*(O/E)*]*) worms to treatment with two oxidative-stress inducing agents, paraquat and *tert*-Butyl hydroperoxide (*t*-BOOH). Paraquat induces cellular damage by elevating intracellular superoxide levels (Hassan and Fridovich, 1979), and *t*-BOOH damages cellular lipids and proteins through peroxidation (Mathews *et al.*, 1994). Under the paraquat or *t*-BOOH conditions where *sir-2.1(ok434)* mutants were sensitive and *hcf-1(pk924)* worms resistant to the treatments, *sir-2.1(ok434) hcf-1(pk924)* worms survived the paraquat or *t*-BOOH exposure as well as *hcf-1(pk924)* single mutants did, and were significantly more resistant than N2 or *sir-2.1(ok434)* worms (Figures 2.1C,E; Figures 2.2A,C; Tables 2.1C,E). Furthermore, overexpressing *sir-2.1* in *hcf-1(pk924)* mutants did not further enhance the paraquat or *t*-BOOH-resistance of *hcf-1(pk924)* worms (Figures 2.1D,F; Figures 2.2B,D; Tables 2.1D,F). Overall, our observations are consistent with a model in which *hcf-1* acts downstream of *sir-2.1* to modulate longevity and oxidative stress responses in *C. elegans*.

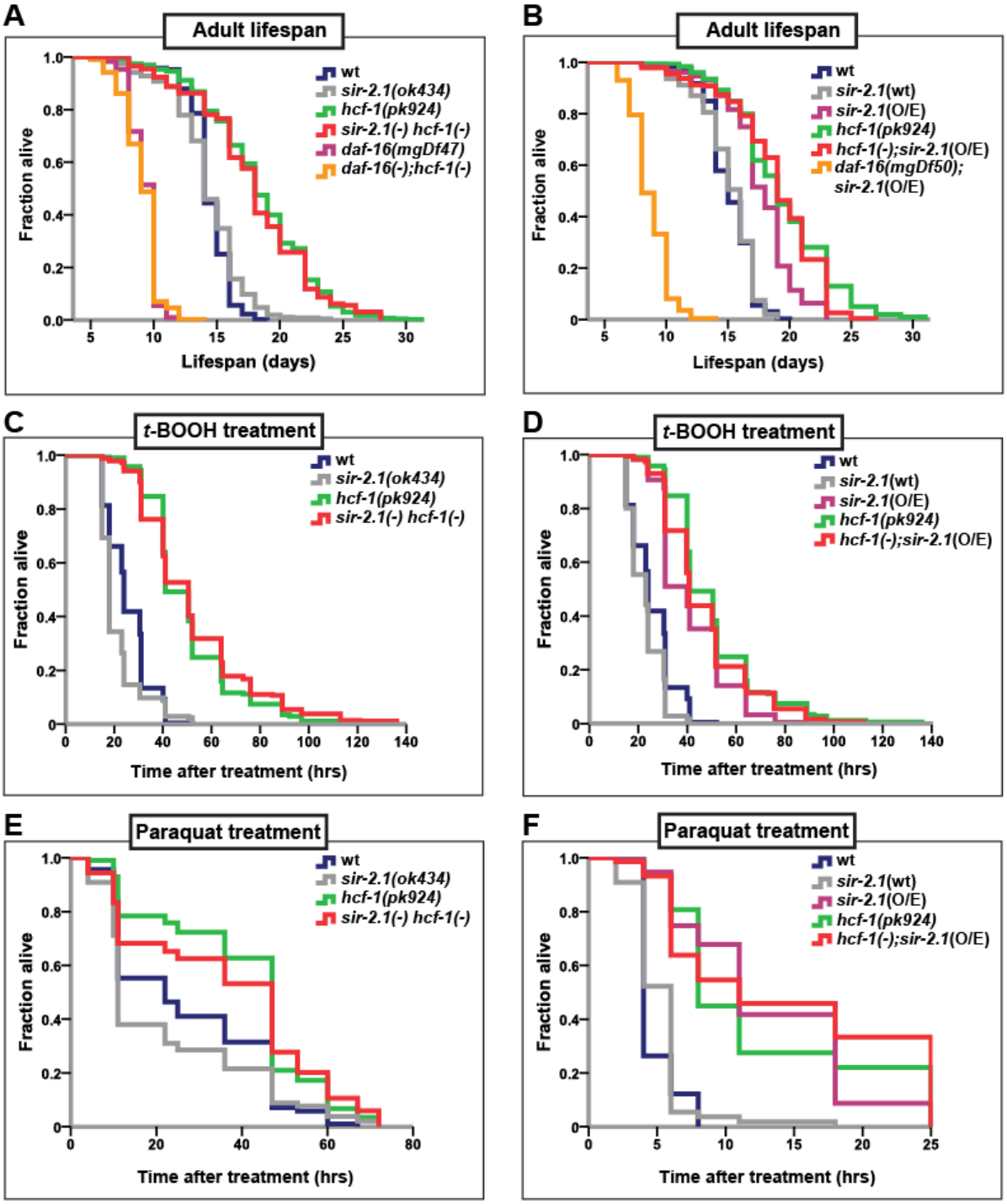
14-3-3 proteins are required for lifespan extension in worms carrying the *hcf-1* mutation

In *C. elegans*, 14-3-3 proteins are required for lifespan extension and stress resistance conferred by extra copies of *sir-2.1*, as well as for facilitating the association of SIR-2.1 and DAF-16 (Berdichevsky *et al.*, 2006; Wang *et al.*, 2006). Our findings that *hcf-1* and *sir-2.1* act together to regulate *daf-16* raise the possibility that *hcf-1* may also functionally interact with *14-3-3*. To address this question, we examined the genetic relationship between *hcf-1* and *14-3-3* in lifespan. The 14-3-3 homologs in *C. elegans* are encoded by two highly similar genes *ftt-2* and *par-5*, which share ~80% sequence identity (Wang and Shakes, 1997). RNAi constructs targeting the coding sequences of *ftt-2* and *par-5* are not specific and will knockdown both genes, whereas RNAi constructs targeting the 3' UTR of each are gene-specific (Figure 2.8A and (Li *et al.*, 2007)). We found that knocking down either *ftt-2* or *par-*

Figure 2.1. *hcf-1* acts downstream of *sir-2.1* to modulate lifespan and oxidative stress response.

(A-B) Lifespans of synchronized adult populations of indicated genotypes. (A) Data pooled from four independent experiments are plotted. One of four *sir-2.1(ok434) hcf-1(pk924)* lines is shown. (See Table 2.1A). (B) Pooled data from three independent experiments are displayed. One of five *hcf-1(pk924);pkIs1642[sir-2.1*(O/E)*]* lines is shown (See Table 2.1B). (C-F) Oxidative stress response of adult worms. (C-D) Day one adult worms were exposed to 6mM *t*-BOOH on plates and their survival monitored through time. The survival curves represent pooled data from two independent experiments. (E-F) Day two adult worms were exposed to 150mM (E) or 200mM (F) paraquat in M9 buffer and their survival monitored through time. Survival curves are generated using pooled data from two independent experiments (E) or data from one of two representative experiments (F). See Tables 2.1A-F for statistics and Figures 2.1C-F for linear mixed model analysis plots.

All lifespan and stress experiments were carried out at 25°C. Quantitative data and statistical analyses are displayed in Tables 2.1A-D.


A
Adult lifespan
Fraction alive
Lifespan (days)
wt
sir-2.1(ok434)
hcf-1(pk924)
sir-2.1(-) hcf-1(-)
daf-16(mgDf47)
daf-16(-);hcf-1(-)
B
Adult lifespan
Fraction alive
Lifespan (days)
wt
sir-2.1(wt)
sir-2.1(O/E)
hcf-1(pk924)
hcf-1(-);sir-2.1(O/E)
daf-16(mgDf50);
sir-2.1(O/E)
C
t-BOOH treatment
Fraction alive
Time after treatment (hrs)
wt
sir-2.1(ok434)
hcf-1(pk924)
sir-2.1(-) hcf-1(-)
D
t-BOOH treatment
Fraction alive
Time after treatment (hrs)
wt
sir-2.1(wt)
sir-2.1(O/E)
hcf-1(pk924)
hcf-1(-);sir-2.1(O/E)
E
Paraquat treatment
Fraction alive
Time after treatment (hrs)
wt
sir-2.1(ok434)
hcf-1(pk924)
sir-2.1(-) hcf-1(-)
F
Paraquat treatment
Fraction alive
Time after treatment (hrs)
wt
sir-2.1(wt)
sir-2.1(O/E)
hcf-1(pk924)
hcf-1(-);sir-2.1(O/E)

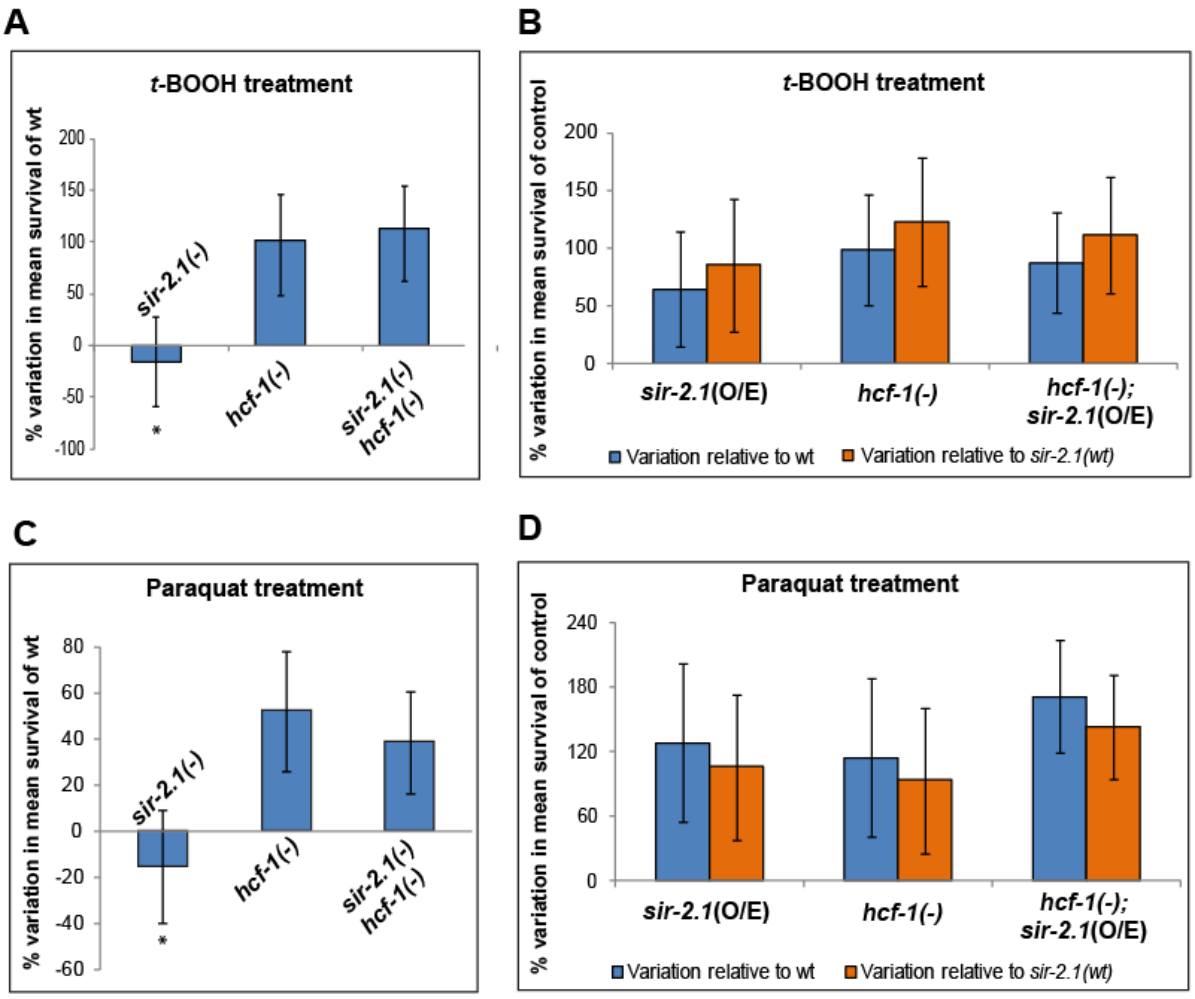


Figure 2.2. *hcf-1* is epistatic to *sir-2.1* in oxidative stress response.

(A-D) The average mean variation in survival of each strain relative to wild-type N2 or *pkIs1641* is displayed. Mean survival is calculated starting from the time of *t*-BOOH (A-B) or paraquat (C-D) exposure. The data represent the pooled data from two independent experiments (* denotes a *p*-value<0.05 compared to *sir-2.1(-) hcf-1(-)*).

All stress experiments were carried out at 25°C. Quantitative data and statistical analyses are displayed in Tables 2.1C-F.

Figure 2.3. Low copy overexpression of *sir-2.1* extends lifespan.

(A) Lifespans of wt, *sir-2.1*(wt) (*pkIs1641), sir-2.1*(O/E) *(pkIs1642), sir-2.*1(wt)-1X (one time outcrossed *pkIs1641*, strain IU91.1), *sir-2.1*(O/E)-1X (one-time outcrossed *pkIs1642*, strain IU94) are displayed. We found that the *sir-2.1* overexpressor strain continues to extend lifespan after outcrossing into our lab N2 strain. See Table 2.1G for quantitative and statistical data. (B) The lifespan extension by *pkIs1642 [sir-2.1*(O/E)*]* is suppressed by *sir-2.1* knockdown as previously reported (Berdichevsky *et al.*, 2006). Lifespans of Strain+RNAi combinations are displayed. To ensure significant knockdown of SIR-2.1, worms were exposed to RNAi for three generations before proceeding with the experiment. See also Table 2.1H. Lifespans were carried out at 25°C. (C) A subpopulation of RNAi-treated worms used in (B) were lysed and analyzed by western blotting to measure SIR-2.1 protein levels in order to confirm efficient knockdown. SIR-2.1 levels are substantially reduced in *sir-2.1* RNAi treated strains. (D) mRNA levels of *sir-2.1* are quantified by RT-qPCR and normalized to *act-1*. Similar to protein levels, mRNA levels of *sir-2.1* are significantly diminished upon RNAi treatment.

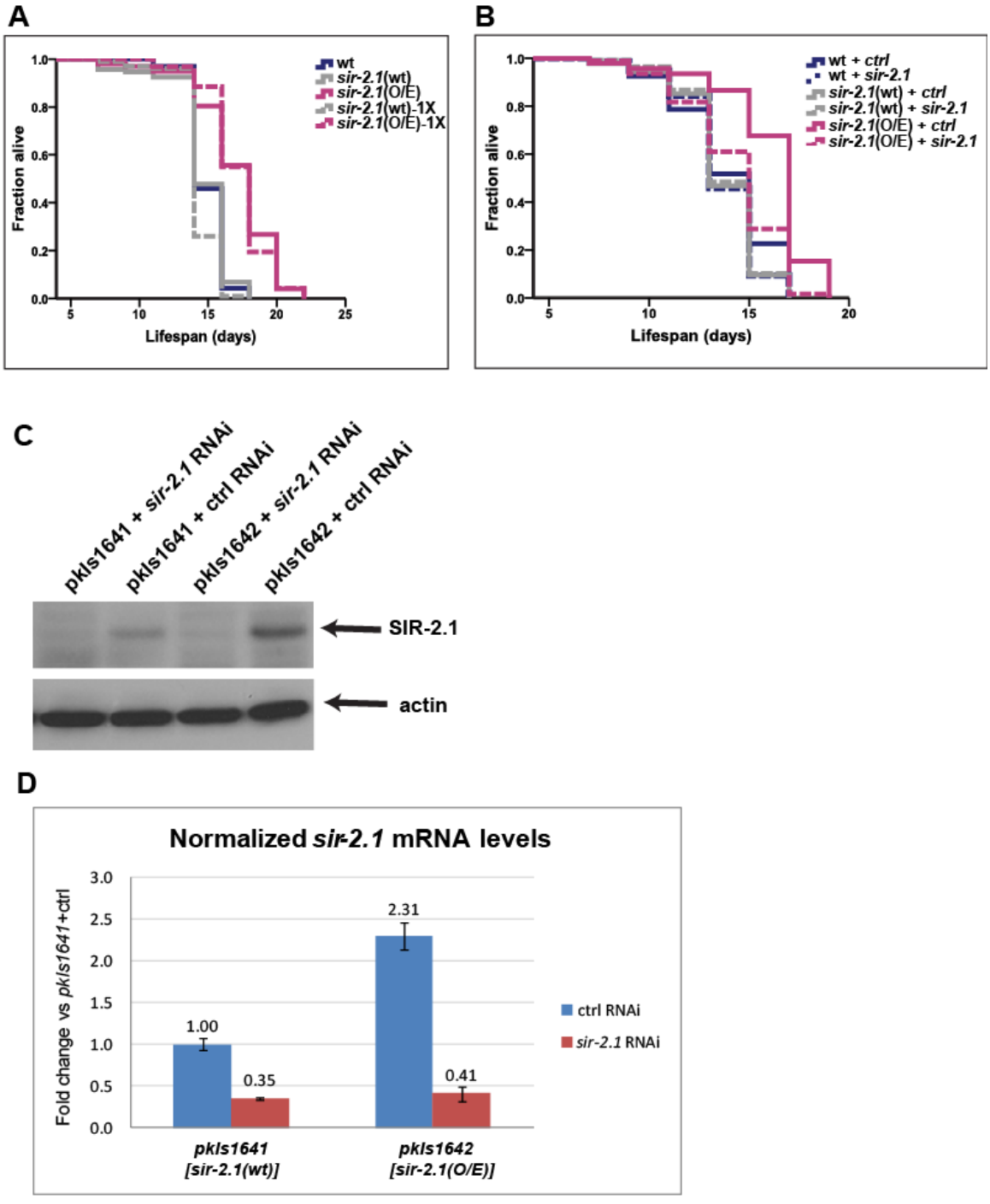

A
Fraction alive
Lifespan (days)
wt
sir-2.1(wt)
sir-2.1(O/E)
sir-2.1(wt)-1X
sir-2.1(O/E)-1X
B
Fraction alive
Lifespan (days)
wt + ctrl
wt + sir-2.1
sir-2.1(wt) + ctrl
sir-2.1(wt) + sir-2.1
sir-2.1(O/E) + ctrl
sir-2.1(O/E) + sir-2.1
C
pkIs1641 + sir-2.1 RNAi
pkIs1641 + ctrl RNAi
pkIs1642 + sir-2.1 RNAi
pkIs1642 + ctrl RNAi
SIR-2.1
actin
D
Normalized sir-2.1 mRNA levels
Fold change vs pkIs1641+ctrl
1.00
0.35
2.31
0.41
ctrl RNAi
sir-2.1 RNAi
pkIs1641
[sir-2.1(wt)]
pkIs1642
[sir-2.1(O/E)]

Table 2.1A

***sir-2.1(ok434) hcf-1(pk924)* double mutant analysis in lifespan**

	Strain	Mean LS + SEM(Days)	Total N	*p*-value vs. N2	*p*-value vs. *hcf-1(pk924)*	*p*-value vs *sir-2.1(ok434)*	% effect on N2
#1	N2	14.3 ± 0.2	102		<0.001	0.007	
	hcf-1(pk924)	17.8 ± 0.5	102	<0.001		<0.001	24
	sir-2.1(ok434)	13.3 ± 0.3	100	<0.001	<0.001		-7
	sir-2.1(-) *hcf-1*(-) (#1)	17.0 ± 0.8	51	<0.001	0.906	<0.001	19
	daf-16(mgDf47)	9.4 ± 0.1	102	<0.001	<0.001	<0.001	-34
	daf-16(-);*hcf-1*(-)	8.7 ± 0.2	92	<0.001	<0.001	<0.001	-39
#2	N2	13.9 ± 0.2	102		<0.001	0.001	
	hcf-1(pk924)	17.5 ± 0.4	114	<0.001		<0.001	26
	sir-2.1(ok434)	12.8 ± 0.2	99	0.001	<0.001		-8
	sir-2.1(-) *hcf-1*(-) (#1)	18.0 ± 0.5	104	<0.001	<0.001	<0.001	30
	daf-16(mgDf47)	9.2 ± 0.1	100	<0.001	<0.001	<0.001	-34
	daf-16(-);*hcf-1*(-)	8.6 ± 0.2	98	<0.001	0.038	<0.001	-38
#3	N2	14.4 ± 0.2	102		<0.001	<0.001	
	hcf-1(pk924)	19.7 ± 0.5	95	<0.001		<0.001	37
	sir-2.1(ok434)	15.6 ± 0.3	100	<0.001	<0.001		8
	sir-2.1(-) *hcf-1*(-) (#1)	20.6 ± 0.8	89	<0.001	0.002	<0.001	43
	sir-2.1(-) *hcf-1*(-) (#2)	18.3 ± 0.5	100	<0.001	0.088	<0.001	27
	sir-2.1(-) *hcf-1*(-) (#3)	21.2 ± 0.7	100	<0.001	0.001	<0.001	47
	sir-2.1(-) *hcf-1*(-) (#4)	20.4 ± 0.6	101	<0.001	0.015	<0.001	42
	daf-16(mgDf47)	9.1 ± 0.1	99	<0.001	<0.001	<0.001	-37
	daf-16(-);*hcf-1*(-)	8.9 ± 0.1	97	<0.001	<0.001	<0.001	-38
#4	N2	14.5 ± 0.2	95		<0.001	<0.001	
	hcf-1(pk924)	18.0 ± 0.3	95	<0.001		<0.001	24
	sir-2.1(ok434)	15.3 ± 0.5	92	<0.001	<0.001		6
	sir-2.1(-) *hcf-1*(-) (#1)	17.4 ± 0.2	92	<0.001	0.859	<0.001	19
	sir-2.1(-) *hcf-1*(-) (#2)	18.8 ± 0.4	97	<0.001	0.569	<0.001	20
	sir-2.1(-) *hcf-1*(-) (#3)	19.3 ± 0.3	71	<0.001	0.056	<0.001	30
	sir-2.1(-) *hcf-1*(-) (#4)	18.2 ± 0.2	105	<0.001	0.002	<0.001	34
	daf-16(mgDf47)	9.2 ± 0.1	106	<0.001	<0.001	<0.001	-37
	daf-16(-);*hcf-1*(-)	10.0 ± 0.1	94	<0.001	<0.001	<0.001	-31

Experiments #1 and #2 were conducted using a lower concentration of bacteria (1X, see Materials and Methods). #3 and #4 were done on 5X OP50 bacteria.

All survival analyses were done using SPSS software using Kaplan Meier analysis and log-rank test to compute *p*-values.

p-value <0.05 is considered statistically significant.

Although the *sir-2.1(ok434)* mutants have been previously reported to exhibit a slightly shorter lifespan than that of wild-type worms, we observed variable results where the mutants would display either slightly shorter or longer lifespans (see above). We note that we carried out the lifespan assays with two slightly different protocols, where bacteria food was slightly more concentrated in two of the experiments (#1&2) (please refer to M&M for details), and that the *sir-2.1(ok434)* mutants tended to live shorter under assaying conditions with lower food and longer on more concentrated bacteria lawns (#3&4). However, whether different bacteria food concentration is the cause of the variability of the *sir-2.1* mutant lifespan needs further investigation in the future. Nevertheless, we found that all four independent lines of the double mutants exhibited lifespans similar to that of *hcf-1(pk924)* single mutant worms, and significantly longer than that of *sir-2.1(ok434)* mutants.

Table 2.1A continued.

Kaplan Meier analysis - experiments pooled							
	Strain	**Mean LS + SEM(Days)**	**Total N**	***p*-value vs. N2**	***p*-value vs. *hcf-1(pk924)***	***p*-value vs. *sir-2.1(ok434)***	**% effect on N2**
1-4	N2	14.3 ± 0.1	400		<0.001	0.012	
	hcf-1(pk924)	18.2 ± 0.2	406	<0.001		<0.001	28
	sir-2.1(ok434)	14.2 ± 0.1	390	0.012	<0.001		0
	sir-2.1(-) *hcf-1*(-) (#1)	18.4 ± 0.3	336	<0.001	0.004	<0.001	28
	sir-2.1(-) *hcf-1*(-) (#2)*	17.9 ± 0.3	196	<0.001	0.556	<0.001	25
	sir-2.1(-) *hcf-1*(-) (#3)	20.2 ± 0.5	170	<0.001	<0.001	<0.001	41
	sir-2.1(-) *hcf-1*(-) (#4)	19.9 ± 0.4	205	<0.001	<0.001	0.001	39
	daf-16(mgDf47)	9.2 ± 0.1	406	<0.001	<0.001	<0.001	-35
	daf-16(-);*hcf-1*(-)	9.1 ± 0.1	380	<0.001	<0.001	<0.001	-37

* Shown in Fig 2.1A.
Data for each strain, except for *sir-2.1*(-) *hcf-1*(-) (#2-4), are pooled from 4 independent experiments. Data for *sir-2.1*(-) *hcf-1*(-) (#2-4) double mutant lines are pooled from two experiments.

Table 2.1B

***pkIs1642[sir-2.1*(O/E)*]* and *hcf-1(pk924)* epistasis analysis in lifespan**

	Strain	Mean LS + SEM(Days)	Total N	*p*-value vs. *hcf-1(pk924)*	*p*-value vs. *pkIs1641*	*p*-value vs *pkIs1642*	% effect on *pkIs1641*
#1	N2	15.4 ± 0.2	94	<0.001	0.008	<0.001	4
	hcf-1(pk924)	18.4 ± 0.4	101		<0.001	0.002	25
	pkIs1641[sir-2.1(wt)*]*	14.7 ± 0.2	94	<0.001		<0.001	
	pkIs1642 [sir-2.1(O/E)*]*	17.5 ± 0.2	104	0.002	<0.001		19
	hcf-1(pk924);pkIs1642 (#1)	17.5 ± 0.4	88	0.122	<0.001	0.584	19
	hcf-1(pk924);pkIs1642 (#2)	19.1 ± 0.4	69	0.401	<0.001	<0.001	30
	hcf-1(pk924);pkIs1642 (#3)	16.2 ± 0.3	110	<0.001	<0.001	0.014	10
	hcf-1(pk924);pkIs1642 (#4)	17.0 ± 0.4	88	0.004	<0.001	0.899	15
	hcf-1(pk924);pkIs1642 (#5)	17.8 ± 0.3	103	0.058	<0.001	0.087	21
	daf-16(mgDf50);pkIs1642	8.5 ± 0.1	97	<0.001	<0.001	<0.001	-42
#2	N2	15.1 ± 0.1	99	<0.001	0.428	<0.001	1
	hcf-1(pk924)	18.9 ± 0.3	96		<0.001	0.001	26
	pkIs1641[sir-2.1(wt)*]*	15.0 ± 0.3	98	<0.001		<0.001	
	pkIs1642 [sir-2.1(O/E)*]*	17.3 ± 0.3	101	0.001	<0.001		15
	hcf-1(pk924);pkIs1642 (#1)	16.7 ± 0.6	58	0.007	<0.001	0.971	11
	hcf-1(pk924);pkIs1642 (#2)	19.1 ± 0.4	58	0.427	<0.001	<0.001	27
	hcf-1(pk924);pkIs1642 (#3)	15.9 ± 0.4	104	<0.001	<0.001	0.002	6
	hcf-1(pk924);pkIs1642 (#4)	16.5 ± 0.4	65	<0.001	0.002	0.056	10
	hcf-1(pk924);pkIs1642 (#5)	17.3 ± 0.3	95	<0.001	<0.001	0.982	15
	daf-16(mgDf50);pkIs1642	8.5 ± 0.1	97	<0.001	<0.001	<0.001	-43
#3	N2	15.0 ± 0.2	98	<0.001	0.727	0.007	0
	hcf-1(pk924)	18.9 ± 0.3	100		<0.001	<0.001	28
	pkIs1641[sir-2.1(wt)*]*	15.4 ± 0.2	102	<0.001		<0.001	
	pkIs1642 [sir-2.1(O/E)*]*	18.1 ± 0.3	94	<0.001	<0.001		17
	hcf-1(pk924);pkIs1642 (#1)	17.1 ± 0.4	80	0.007	<0.001	0.573	13
	hcf-1(pk924);pkIs1642 (#2)	18.4 ± 0.5	80	0.087	<0.001	<0.001	25
	hcf-1(pk924);pkIs1642 (#3)	15.5 ± 0.3	107	<0.001	<0.001	<0.001	5
	hcf-1(pk924);pkIs1642 (#4)	17.5 ± 0.3	96	<0.001	<0.002	<0.001	9
	hcf-1(pk924);pkIs1642 (#5)	17.5 ± 0.3	96	<0.001	<0.001	0.924	16
	daf-16(mgDf50);pkIs1642	9.1 ± 0.1	87	<0.001	<0.001	<0.001	-42

All survival analyses were done using SPSS software, Kaplan Meier analysis and log-rank test to compute *p*-values. *p*-value <0.05 is considered statistically significant.
Experiments were conducted using 5X concentrated OP50 bacteria.
hcf-1(pk924);pkIs1642 (#1-5) represent independent isolates obtained from a cross.
All survival analyses were done using SPSS software, Kaplan Meier analysis and log-rank test to compute *p*-values. *p*-value <0.05 is considered statistically significant.

Kaplan Meier analysis - experiments pooled

	Strain	Mean LS + SEM(Days)	Total N	*p*-value vs. *hcf-1(pk924)*	*p*-value vs. *pkIs1641*	*p*-value vs *pkIs1642*	% effect on *pkIs1641*
1-3	N2	15.1 ± 0.1	292	<0.001	0.727	0.007	0
	hcf-1(pk924)	19.4 ± 0.2	305		<0.001	<0.001	28
	pkIs1641[sir-2.1(wt)*]*	15.1 ± 0.1	294	<0.001		<0.001	
	pkIs1642 [sir-2.1(O/E)*]*	17.6 ± 0.2	299	<0.001	<0.001		17
	hcf-1(pk924);pkIs1642 (#1)	17.1 ± 0.3	226	<0.001	<0.001	0.573	13
	hcf-1(pk924);pkIs1642 (#2)*	18.9 ± 0.2	240	0.087	<0.001	<0.001	25
	hcf-1(pk924);pkIs1642 (#3)	15.9 ± 0.2	321	<0.001	<0.001	<0.001	5
	hcf-1(pk924);pkIs1642 (#4)	16.5 ± 0.2	253	<0.001	<0.001	<0.001	9
	hcf-1(pk924);pkIs1642 (#5)	17.5 ± 0.2	294	<0.001	<0.001	<0.001	16
	daf-16(mgDf50);pkIs1642	8.7 ± 0.1	285	<0.001	<0.001	0.924	-42

* Shown in Fig 2.1B
Data for each strain are pooled from 3 independent experiments.

Table 2.1C

***sir-2.1(ok434)* and *hcf-1(pk924)* epistasis analysis in *t*-Butyl hydroperoxide**

Kaplan Meier analysis

Experiment	Strain	Mean survival + SEM(Hrs)	Total N	% effect on N2
#1	N2	26.2 ± 0.6	127	
	hcf-1(pk924)	41.8 ± 1.2	105	59.8
	sir-2.1(ok434)	19.0 ± 0.5	109	-27.2
	sir-2.1(-) *hcf-1*(-) (#1)	43.4 ± 1.2	105	66.1
	sir-2.1(-) *hcf-1*(-) (#2)	40.2 ± 1.3	107	53.5
#2	N2	24.9 ± 0.9	113	
	hcf-1(pk924)	58.7 ± 2.0	108	136.3
	sir-2.1(ok434)	23.9 ± 1.1	103	-3.7
	sir-2.1(-) *hcf-1*(-) (#1)	73.2 ± 2.7	88	194.5
	sir-2.1(-) *hcf-1*(-) (#2)	55.0 ± 2.1	114	121.4

Linear Mixed model analysis

#1 and #2 (pooled)(Fig 2.2A)	Strain	Averaged % variation compared to wt ± SEM	Total N	*p*-value vs. *sir-2.1(-) hcf1(-)*
	sir-2.1(ok434)	-15.5 ± 43.1	212	0.035
	hcf-1(pk924)	98.0 ± 48.9	213	0.715
	sir-2.1(-) *hcf-1*(-)	108.9 ± 46.3	414	

Survival of worms treated with 6mM *t*-BOOH was monitored. All survival analyses were done using SPSS software, Kaplan Meier analysis. Linear Mixed model analysis was used to calculate the averaged percent variation relative to wt. Linear Mixed model analysis allows statistical evaluation of differences between various treatments (mutants) by taking into account the experimental variation. *p*-value<0.05 is considered significantly different from control.

Kaplan Meier analysis - experiments and double mutants pooled

Experiment	Strain	Mean survival + SEM(Hrs)	Total N	*p*-value vs. N2	*p*-value vs. *hcf-1(pk924)*	% effect on N2
1+2 (Fig 2.1C)	N2	25.5 ± 0.5	240		<0.001	
	hcf-1(pk924)	50.1 ± 1.3	212	<0.001		96.0
	sir-2.1(ok434)	21.4 ± 0.6	213	<0.001	<0.001	-16.4
	sir-2.1(-) *hcf-1*(-)	52.2 ± 1.1	414	<0.001	0.193	104.3

Data from two independent experiments as well as two genotypically identical *sir-2.1(-) hcf-1(-)* double mutants were pooled and analyzed using Kaplan Meier and log-rank statistics. *p*-value<0.05 is considered significantly different from the corresponding control.

Table 2.1D

***pkIs1642[sir-2.1*(O/E)] and *hcf-1(pk924)* epistasis analysis in *t*-Butyl hydroperoxide**

Kaplan Meier analysis

Experiment	Strain	Mean survival + SEM(Hrs)	Total N	% effect on N2	% effect on *pkIs1641*
#1	N2	26.2 ± 0.6	127		11.4
	pkIs1641[sir-2.1(wt)*]*	23.5 ± 0.6	100	-10.2	
	hcf-1(pk924)	41.8 ± 1.2	105	59.8	32.0
	pkIs1642 [sir-2.1(O/E)*]*	31.0 ± 0.4	118	18.5	78.0
	hcf-1(pk924);pkIs1642 (#1)	32.3 ± 0.6	115	23.4	37.5
	hcf-1(pk924);pkIs1642 (#2)	43.0 ± 1.1	108	64.3	83.0
#2	N2	24.9 ± 0.9	113		13.3
	pkIs1641[sir-2.1(wt)*]*	21.9 ± 0.7	101	-11.8	
	hcf-1(pk924)	58.7 ± 2.0	108	136.3	167.7
	pkIs1642 [sir-2.1(O/E)*]*	52.3 ± 1.2	109	110.4	138.4
	hcf-1(pk924);pkIs1642 (#1)	46.2 ± 1.2	103	86.1	110.8
	hcf-1(pk924);pkIs1642 (#2)	68.6 ± 1.7	102	176.0	212.7

Linear Mixed model analysis

Pooled #1,2 (Fig 2.2B)	Strain	Averaged % variation compared to wt ± SEM	Averaged % variation compared to *pkIs1641* ± SEM	Total N	*p*-value vs. *hcf-1(pk924); pkIs1642* (wt / *pkIs1641)*
	*pkIs1642[sir-2.1(*O/E)*]*	64.4 ± 50.1	85.2 ± 57.9	227	0.557 / 0.560
	hcf-1(pk924)	98.0 ± 47.9	122.9 ± 55.5	213	0.782 / 0.784
	hcf-1(pk924);pkIs1642	87.4 ± 43.4	111.0 ± 50.4	428	

Survival of worms treated with 6mM *t*-BOOH was monitored. All survival analyses were done using SPSS software, Kaplan Meier analysis. We used Linear Mixed model analysis to calculate the averaged percent variation relative to wt or *pkIs1641*. *p*-value<0.05 is considered significantly different from control.

Kaplan Meier analysis - experiments and double mutants pooled

Experiment	Strain	Mean survival + SEM(Hrs)	Total N	*p*-value vs. N2	*p*-value vs. *hcf-1(pk924)*	% effect on N2
#1 + 2 (Fig 2.1D)	N2	25.5 ± 0.5	240		<0.001	
	pkIs1641[sir-2.1(wt)*]*	22.7 ± 0.5	201	<0.001	<0.001	-11.1
	hcf-1(pk924)	50.1 ± 1.3	213	<0.001		96.0
	pkIs1642 [sir-2.1(O/E)*]*	41.3 ± 1.0	227	<0.001	<0.001	61.5
	hcf-1(pk924);pkIs1642	47.0 ± 0.9	428	<0.001	0.093	83.9

Data from two two genotypically identical *hcf-1(-);pkIs1642[sir-2.1*(O/E)*]* strains were pooled and analyzed using Kaplan Meier and log-rank statistics. *p*-value<0.05 is considered significantly different from corresponding control.

Table 2.1E

***sir-2.1(ok434)* and *hcf-1(pk924)* epistasis analysis in paraquat**

Kaplan Meier analysis

Experiment	Strain	Mean survival + SEM(Hrs)	Total N	% effect on N2
#1	N2	21.0 ± 1.2	156	
	hcf-1(pk924)	35.9 ± 1.4	177	71.0
	sir-2.1(ok434)	18.6 ± 1.3	193	-11.1
	sir-2.1(-) *hcf-1*(-) (#1)	37.3 ± 1.8	152	77.8
	sir-2.1(-) *hcf-1*(-) (# 2)	29.3 ± 2.0	135	39.8
	sir-2.1(-) *hcf-1*(-) (# 3)	34.9 ± 1.7	157	66.2
	sir-2.1(-) *hcf-1*(-) (# 4)	36.5 ± 1.5	183	74.0
#2	N2	33.5 ± 1.1	281	
	hcf-1(pk924)	44.6 ± 1.0	277	33.2
	sir-2.1(ok434)	26.9 ± 1.1	295	-19.7
	sir-2.1(-) *hcf-1*(-) (# 1)	30.4 ± 1.9	116	-9.2
	sir-2.1(-) *hcf-1*(-) (# 2)	39.4 ± 1.3	303	17.6
	sir-2.1(-) *hcf-1*(-) (# 3)	40.9 ± 2.0	129	22.0
	sir-2.1(-) *hcf-1*(-) (# 4)	40.3 ± 0.8	549	20.3

Linear Mixed model analysis

#1 and #2 (pooled)(Fig 2.2C)	Strain	Averaged % variation compared to wt ± SEM	Total N	*p*-value vs. *sir-2.1(-) hcf1(-)*
	sir-2.1(ok434)	-15.4 ± 24.4	488	0.007
	hcf-1(pk924)	52.1 ± 26.3	454	0.296
	sir-2.1(-) *hcf-1*(-)	38.6 ± 22.1	1725	

Survival of worms treated with 150mM paraquat in M9 buffer was monitored. All survival analyses were done using SPSS software, Kaplan Meier analysis. We used Linear Mixed model analysis to calculate the averaged percent variation relative to wt. *p*-value<0.05 is considered significantly different from control.

Kaplan Meier analysis - experiments and double mutants pooled

Experiment	Strain	Mean survival + SEM(Hrs)	Total N	*p*-value vs. N2	*p*-value vs. *hcf-1(pk924)*	% effect on N2
1+2 (Fig 2.1E)	N2	21.0 ± 1.2	295		<0.001	
	hcf-1(pk924)	35.9 ± 1.4	314	0.017		47.5
	sir-2.1(ok434)	18.6 ± 1.3	339	<0.001	<0.001	-17.4
	sir-2.1(-) *hcf-1*(-)	37.3 ± 1.8	1079	<0.001	0.686	36.7

Data from two independent experiments as well as four genotypically identical *sir-2.1(-) hcf-1(-)* double mutants were pooled and analyzed using Kaplan Meier and log-rank statistics. *p*-value<0.05 is considered significantly different from the corresponding control.

Table 2.1F

***pkIs1642[sir-2.1*(O/E)] and *hcf-1(pk924)* epistasis analysis in paraquat**

Kaplan Meier analysis

Experiment	Strain	Mean survival + SEM(Hrs)	Total N	% effect on N2	% effect on *pkIs1641*
#1	N2	4.8 ± 0.1	114		-8.4
	pkIs1641[sir-2.1(wt)*]*	5.2 ± 0.2	109	9.2	
	hcf-1(pk924)	12.3 ± 0.7	109	157.8	136.1
	pkIs1642 [sir-2.1(O/E)*]*	13.0 ± 0.5	115	171.5	148.6
	hcf-1(pk924); pkIs1642 (#1)	9.2 ± 0.6	98	93.7	77.4
	hcf-1(pk924);pkIs1642 (#2)	18.8 ± 0.7	109	294.7	261.4
#2	N2	5.5 ± 0.2	116		-12.3
	pkIs1641[sir-2.1(wt)*]*	6.3 ± 0.3	117	14.1	
	hcf-1(pk924)	9.5 ± 0.4	120	71.0	49.9
	pkIs1642 [sir-2.1(O/E)*]*	10.3 ± 0.5	115	84.8	62.0
	hcf-1(pk924);pkIs1642 (#1)	8.5 ± 0.5	110	53.3	34.4
	hcf-1(pk924);pkIs1642 (#2)	18.9 ± 0.6	115	240.3	198.4

Linear Mixed model analysis

	Strain	Averaged % variation compared to wt ± SEM	Averaged % variation compared to *pkIs1641* ± SEM	Total N	*p*-value vs. *hcf-1(pk924); pkIs1642* (wt / *pkIs1641)*
Pooled #1,2 (Fig 2.2D)					
	pkIs1642[sir-2.1(O/E)*]*	128.2 ± 73.9	105.3 ± 68.0	230	0.664 / 0.581
	hcf-1(pk924)	114.4 ± 73.9	93.0 ± 67.9	229	0.569 / 0.675
	hcf-1(pk924);pkIs1642	170.5 ± 52.3	142.9 ± 48.1	432	

Survival of worms treated with 200mM paraquat in M9 buffer was monitored. All survival analyses were done using SPSS software, Kaplan Meier analysis. Linear Mixed model analysis was used to calculate the averaged percent variation relative to wt or *pkIs1641*. *p*-value<0.05 is considered significantly different from control.

Kaplan Meier analysis - experiments and double mutants pooled

Experiment	Strain	Mean survival + SEM(Hrs)	Total N	*p*-value vs. N2	*p*-value vs. *hcf-1(pk924)*	% effect on N2
1+2 (Fig 2.1F)	N2	4.8 ± 0.1	114		<0.001	
	pkIs1641[sir-2.1(wt)*]*	5.2 ± 0.2	109	0.105	<0.001	9.2
	hcf-1(pk924)	12.3 ± 0.7	109	<0.001		157.8
	pkIs1642 [sir-2.1(O/E)*]*	13.0 ± 0.5	115	<0.001	0.831	171.5
	hcf-1(pk924); pkIs1642	14.3 ± 0.6	207	<0.001	0.116	199.6

Data from two genotypically identical *hcf-1(-);pkIs1642[sir-2.1*(O/E)*]* strains were pooled and analyzed using Kaplan Meier and log-rank statistics. *p*-value<0.05 is considered significantly different from the corresponding control.

Table 2.1G

Non-outcrossed and outcrossed *pkIs1642 [sir-2.1*(O/E)*]* lifespan analysis

Strain	Mean LS + SEM(Days)	Total N	*p*-value vs. N2	*p*-value vs. *sir-2.1*(wt)	*p*-value vs *sir-2.1*(wt)-1X	% effect on N2
N2	14.9 ± 0.1	97		0.832	0.003	
pkIs1641[sir-2.1(wt)*]*	14.7 ± 0.2	93	0.832		0.004	-2
pkIs1642 [sir-2.1(O/E)*]*	17.1 ± 0.3	97	<0.001	<0.001	<0.001	14
sir-2.1(wt)-1X	14.3 ± 0.1	100	0.003	0.004		-4
sir-2.1(O/E)-1X	17.2 ± 0.2	95	<0.001	<0.001	<0.001	16

Graph shown in Figure 2.2A.
sir-2.1(wt)-1X (one-time outcrossed *pkIs1641*), *sir-2.1*(O/E)-1X (one-time outcrossed *pkIs1642).* See Table 2.2C for a repeat of the lifespan of outcrossed *sir-2.1* control and O/E strains.
All survival analyses were done using SPSS software, Kaplan Meier analysis and log-rank test to compute *p*-values.
p-value<0.05 is considered statistically significant.

Table 2.1H

***sir-2.1* RNAi knockdown lifespan analysis**

Strain + RNAi	Mean LS + SEM(Days)	Total N	*p*-value vs. N2	*p*-value vs. *sir-2.1*(wt) + *ctrl* RNAi	*p*-value vs *sir-2.1*(wt) + *sir-2.1* RNAi	% effect on N2+ *ctrl*
N2 + *ctrl*	13.8 ± 0.3	95		0.200	0.314	
N2 + *sir-2.1*	13.7 ± 0.2	96	0.144	0.897	0.669	-1
sir-2.1(wt) + *ctrl*	13.7 ± 0.2	94	0.200		0.767	-1
sir-2.1(wt) + *sir-2.1*	13.8 ± 0.2	87	0.314	0.767		0
sir-2.1(O/E)+ *ctrl*	16.1 ± 0.3	96	<0.001	<0.001	<0.001	16
sir-2.1(O/E) + *sir-2.1*	14.3 ± 0.3	94	0.192	0.009	0.020	3

Graph shown in Figure 2.2B.
Worms were grown on RNAi bacteria for 3 generations. This experiment is done once.
All survival analyses were done using SPSS software, Kaplan Meier analysis and log-rank test to compute *p*-values.
p-value <0.05 is considered statistically significant.

5 alone did not substantially reduce *hcf-1(pk924)* lifespan, yet simultaneously diminishing the function of both genes through the non-specific RNAi completely abrogated the longevity effect of *hcf-1* inactivation (Figures 2.4A,B; Tables 2.2A,B). The RNAi data are corroborated by our findings that a null mutation of *ftt-2*, *n4426*, was only able to slightly decrease the lifespan of *hcf-1* mutants (Figure 2.4D; Table 2.2D). As a control, we repeated previously published results (Berdichevsky *et al.*, 2006) where *ftt-2* knockdown suppresses the long-lifespan of the *pkIs1642[sir-2.1*(O/E)*]* strain but not that of *daf-2(e1370)* mutant (Figures 2.5B,C; Table 2.2C). Therefore, we conclude that both *14-3-3* genes are necessary for the longevity increase conferred by *hcf-1* mutation and likely act downstream of *hcf-1*.

hcf-1 and *sir-2.1* co-regulate a specific subset of DAF-16 transcriptional targets important for longevity, cellular detoxification, and fatty acid/lipid/amino acid metabolism

DAF-16 responds to different upstream stimuli by selectively activating and repressing groups of target genes, and hence ensuring appropriate responses to specific signals (Lee *et al.*, 2003; McElwee *et al.*, 2003; Murphy *et al.*, 2003). We previously proposed that *C. elegans* HCF-1 acts as a specificity factor for DAF-16 and negatively regulates DAF-16 on a select set of its target genes (Li *et al.*, 2008). Similarly, *C. elegans* SIR-2.1 is thought to promote DAF-16 regulation of a subset of transcriptional targets (Berdichevsky *et al.*, 2006). As our genetic data suggest that *hcf-1* and *sir-2.1* act in the same genetic pathway to modulate longevity in a *daf-16*-dependent manner, we hypothesized that *hcf-1* inactivation and *sir-2.1* overexpression would have similar effects on DAF-16-mediated transcription. To test this hypothesis, we compared the *daf-16*-dependent global transcriptional changes occurring in the long-lived *hcf-1(pk924)* mutant to those occurring in the long-lived *sir-2.1* overexpressor strain.

We identified the genes whose expression was changed in *hcf-1(pk924)* mutants in a

daf-16-dependent manner by comparing the expression profiles of synchronized *hcf-1(pk924)* mutants to those of *daf-16(mgDf47);hcf-1(pk924)* double mutants using Agilent *C. elegans* gene expression microarrays. In addition, to pinpoint the genes that are responsive to the *hcf-1(pk924)* mutation, instead of those that show expression changes simply due to *daf-16* deletion, we focused on genes that showed a similar trend of expression change both in the *hcf-1(pk924)* vs. N2 and *hcf-1(pk924)* vs. *daf-16(mgDf47);hcf-1(pk924)* comparisons (henceforth referred to as *hcf-1(-)* profile). Likewise, the genes which were differentially regulated by DAF-16 in response to *sir-2.1* overexpression were identified by comparing the strains *pkIs1642[sir-2.1*(O/E)*]* to *daf-16(mgDf50);pkIs1642[sir-2.1*(O/E)*]* and *pkIs1642 [sir-2.1*(O/E)*]* to its transgenic control *pkIs1641[sir-2.1*(wt)*]* (henceforth referred to as *sir-2.1*(O/E) profile). To identify the genes that show consistent and significant expression changes across the independent biological replicates of *hcf-1(-)* or *sir-2.1*(O/E), we used Significance Analysis of Microarrays (SAM) (Tusher *et al.*, 2001) with a stringent criteria of expected false discovery rate (FDR) set at 0%. SAM analysis identified 1,032 significantly affected genes in *hcf-1(-)* and 1,042 genes in *sir-2.1*(O/E) (Figure 2.6A). Next, we compared the two datasets to determine the extent of overlap. Strikingly, we found 866 genes (473 upregulated and 390 downregulated) whose expression changed similarly in *hcf-1(-)* and *sir-2.1*(O/E) profiles, suggesting that the vast majority (>80%) of the genes regulated by DAF-16 in response to *hcf-1* inactivation or *sir-2.1* activation are shared (Figure 2.6B). Of the genes that were expressed in a dissimilar manner between *hcf-1(-)* and *sir-2.1*(O/E) profiles, ~10% displayed an opposite expression change and ~10% were unique to either *hcf-1(-)* or *sir-2.1*(O/E) (Figure 2.6A,B). The finding that the transcriptional outcomes conferred by DAF-16 in response to *hcf-1* mutation or *sir-2.1* overexpression are largely similar corroborates our genetic data suggesting that SIR-2.1 and HCF-1 act in the same pathway to regulate DAF-16. In addition to being regulated by SIR-2.1 and HCF-1, DAF-16 activity is also controlled by the insulin/IGF-1 signaling (IIS) pathway. In response to reduced IIS, DAF-16 translocates

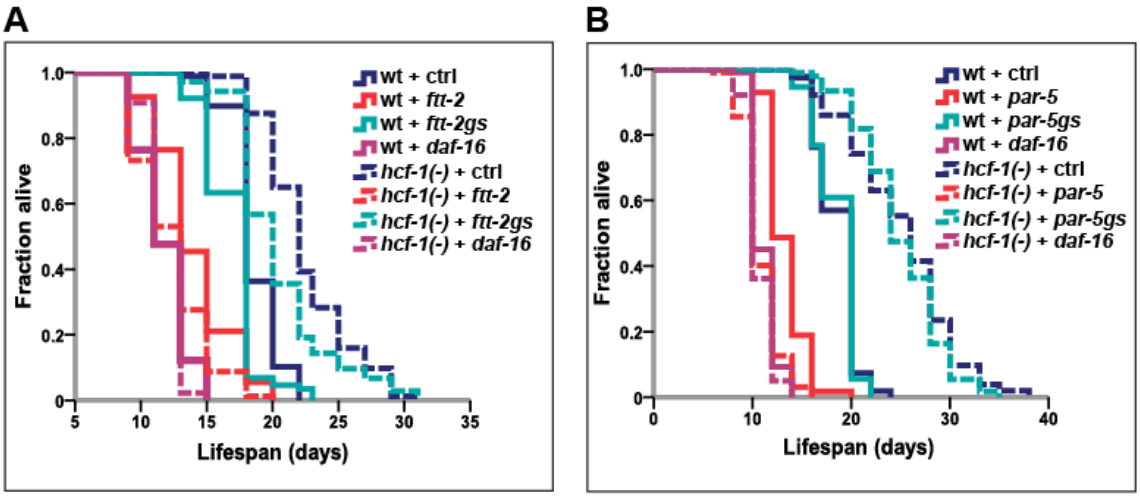


Figure 2.4. *14-3-3* are required for lifespan extension conferred by *hcf-1(pk924)* mutation.

(A-B) Worms were grown on vector, *daf-16, ftt-2* (Ahringer - contains multiple stretches of identical sequences to *par-5*), *par-5* (Ahringer - contains overlapping sequences with *ftt-2*) (Li *et al.*, 2007), *ftt-2gs* (gene specific RNAi targeting 3' UTR of *ftt-2),* or *par-5gs* (gene-specific RNAi targeting 3' UTR of *par-5*) (Li *et al.*, 2007) from egglay until the end of life. The lifespan experiments were carried out at 20°C. Quantitative data and statistical analyses are included in Tables 2.2A,B.

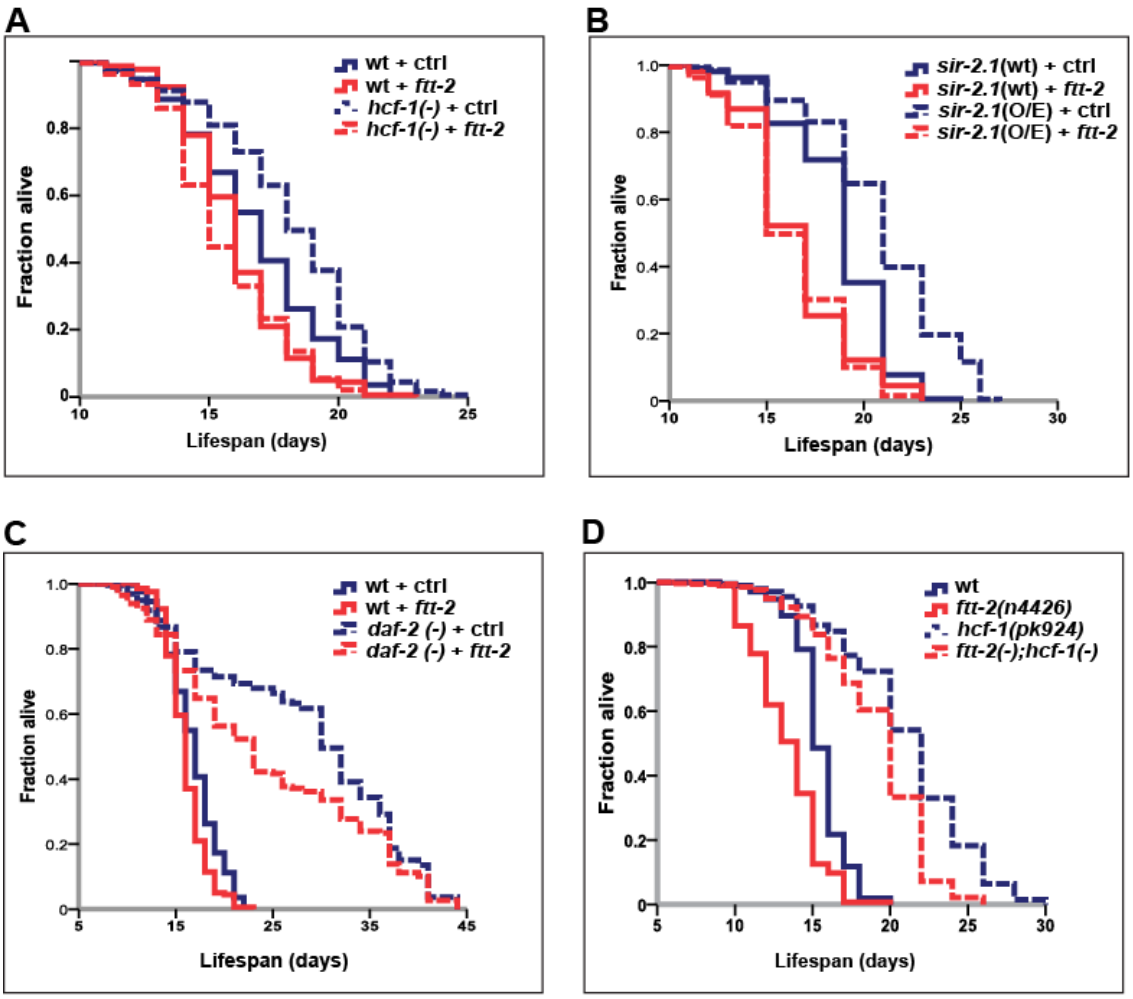


Figure 2.5. *14-3-3* knockdown suppresses lifespan increase by *hcf-1(pk924)* mutation and *sir-2.1* overexpression but not by *daf-2(e1370)* mutation.

(A-C) Worms of indicated genotypes were grown on vector (control) RNAi bacteria at 16°C until young adulthood and subsequently transferred to either control or *ftt-2* (Ahringer) RNAi at 25°C for the remainder of the experiment. Pooled data from two independent experiments are shown. See Table 2.2C for quantitative data. (D) Worms of indicated genotypes were grown on OP50 bacteria at 25°C throughout the experiment and pooled data from two independent experiments are shown (Also see Table 2.2D)

Table 2.2A

***ftt-2* or *ftt-2* gene specific RNAi and *hcf-1(pk924)* epistasis analysis in lifespan**

Strain	RNAi	Mean LS + SEM(Days)	Total N	% lifespan extension by *hcf-1(-)*+RNAi vs. corresponding N2+RNAi	*p*-value vs. N2+vector	*p*-value vs. *hcf-1(-)* + vector RNAi
N2	vector	18.6 ± 0.2	90			<0.001
	ftt-2	14.0 ± 0.3	95		<0.001	<0.001
	ftt-2gs	17.0 ± 0.2	89		<0.001	<0.001
	daf-16	11.7 ± 0.2	93		<0.001	<0.001
hcf-1(pk924)	vector	22.5 ± 0.4	98	21.0	<0.001	
	ftt-2	12.4 ± 0.3	97	-11.9	<0.001	<0.001
	ftt-2gs	20.5 ± 0.4	106	20.4	<0.001	<0.001
	daf-16	11.8 ± 0.1	109	0.7	<0.001	<0.001

Graph shown in Figure 2.4A.
ftt-2 (*ftt-2*-targeting RNAi construct with overlap to *par-5* sequence), *ftt-2gs*= Gene-specific *ftt-2* RNAi
Experiment was carried out at 20°C once.
All survival analyses were performed using SPSS software Kaplan Meier analysis and log-rank test to compute *p*-values. *p*-value<0.05 is considered statistically significant.

Table 2.2B

***par-5* or *par-5* gene specific RNAi and *hcf-1(pk924)* epistasis analysis in lifespan**

Strain	RNAi	Mean LS + SEM(Days)	Total N	% lifespan extension by *hcf-1(-)* +RNAi vs. corresponding N2+RNAi	*p*-value vs. N2+vector	*p*-value vs. *hcf-1(-)* + vector RNAi
N2	vector	18.6 ± 0.2	120			<0.001
	par-5	13.3 ± 0.2	114		<0.001	<0.001
	par-5gs	18.6 ± 0.2	115		<0.001	<0.001
	daf-16	11.1 ± 0.1	103		<0.001	<0.001
hcf-1(pk924)	vector	25.0 ± 0.6	97	34.6	<0.001	
	par-5	10.8 ± 0.2	97	-18.6	<0.001	<0.001
	par-5gs	25.0 ± 0.5	92	34.3	<0.001	0.645
	daf-16	10.7 ± 0.2	91	-3.6	<0.001	<0.001

Graph shown in Figure 2.4B.
par-5 (*par-5*-targeting RNAi construct with overlap to *ftt-2* sequence), *par-5gs*= Gene-specific *par-5* RNAi.
Experiment was carried out at 20°C once.
All survival analyses were performed using SPSS software Kaplan Meier analysis and log-rank test to compute *p*-values. *p*-value ≤0.05 is considered statistically significant.

Table 2.2C

***ftt-2* RNAi and *hcf-1(pk924), sir-2.1*(O/E), or *daf-2(e1370)* epistasis analysis in lifespan**

	Strain	RNAi	Mean LS + SEM(Days)	Total N	% lifespan extension on vector RNAi	% lifespan extension on *ftt-2* RNAi	*p*-value vs. control+ RNAi
#1	N2	vector	16.7 ± 0.3	103			
		ftt-2	15.8 ± 0.2	100			
	hcf-1(pk924)	vector	18.3 ± 0.3	103	9 (vs. N2)		<0.001[a]
		ftt-2	15.6 ± 0.2	106		-1	0.885[b]
	pkIs1641[sir-2.1(wt)*]*	vector	18.7 ± 0.3	107			
		ftt-2	16.0 ± 0.2	111			
	pkIs1642[sir-2.1(O/E)*]*	vector	21.0 ± 0.3	107	12 (vs. pkIs1641)		<0.001[c]
		ftt-2	16.6 ± 0.3	112		-4	0.050[d]
	daf-2(e1370)	vector	30.3 ± 1.1	100	81 (vs. N2)		<0.001[a]
		ftt-2	26.9 ± 1.2	99		71	<0.001[b]
#2	N2	vector	16.8 ± 0.2	104			
		ftt-2	16.3 ± 0.2	108			
	hcf-1(pk924)	vector	18.0 ± 0.3	104	7 (vs. N2)		0.002[a]
		ftt-2	15.6 ± 0.3	102		-4	0.070[b]
	pkIs1641[sir-2.1(wt)*]*	vector	19.0 ± 0.3	107			
		ftt-2	17.1 ± 0.3	105			
	pkIs1642[sir-2.1(O/E)*]*	vector	20.9 ± 0.4	95	10 (vs. pkIs1641)		<0.001[c]
		ftt-2	16.1 ± 0.3	104		-6	0.013[d]
	daf-2(e1370)	vector	26.1 ± 1.0	93	55 (vs. N2)		<0.001[a]
		ftt-2	22.6 ± 0.8	103		38	<0.001[b]

Data from two independent experiments are pooled. Survival plots: Figures S2A,B,C. *sir-2.1*(wt) and *sir-2.1*(O/E) strains are 1X outcrossed in our lab.
Experiment was carried out at 25°C.
All survival analyses were performed using SPSS software Kaplan Meier analysis and log-rank test to compute *p*-values. *p*-value<0.05 is considered statistically significant.
[a]*p*-value vs. N2+vector, [b]*p*-value vs. N2+*ftt-2* RNAi, [c]*p*-value vs. *pkIs1641*+vector, [d]*p*-value vs. *pkIs1641+ftt-2* RNAi

Kaplan Meier Analysis - experiments pooled

	Strain	RNAi	Mean LS + SEM(Days)	Total N	% lifespan extension on vector RNAi	% lifespan extension on *ftt-2* RNAi	*p*-value vs. control+ RNAi
1+2	N2	vector	16.8 ± 0.2	207			
(Figure 2.5A-C)		*ftt-2*	16.1 ± 0.1	208			
	hcf-1(pk924)	vector	18.1 ± 0.2	207	8 (vs. N2)		<0.001[a]
		ftt-2	15.6 ± 0.2	208		-3	0.139[b]
	pkIs1641[sir-2.1(wt)*]*	vector	18.9 ± 0.2	214			
		ftt-2	16.5 ± 0.2	216			
	pkIs1642[sir-2.1(O/E)*]*	vector	20.9 ± 0.2	202	11 (vs. pkIs1641)		<0.001[c]
		ftt-2	16.3 ± 0.2	216		-1	0.529[d]
	daf-2(e1370)	vector	28.4 ± 0.7	198	69 (vs. N2)		<0.001[a]
		ftt-2	24.5 ± 0.7	199		50	0.007[b]

Data from two independent experiments are pooled. *sir-2.1*(wt) and *sir-2.1*(O/E) strains are 1X outcrossed in our lab.
[a]*p*-value vs. N2+vector, [b]*p*-value vs. N2+*ftt-2* RNAi, [c]*p*-value vs. *pkIs1641*+vector, [d]*p*-value vs. *pkIs1641+ftt-2* RNAi

Table 2.2D

***ftt-2(n4426)* and *hcf-1(pk924)* epistasis analysis in lifespan**

	Strain	Mean LS + SEM(Days)	Total N	% lifespan extension vs. N2	% lifespan extension vs. *ftt-2(-)*	*p*-value vs. N2
#1	N2	15.8 ± 0.2	107			
	hcf-1(pk924)	22.2 ± 0.4	105	41		<0.001
	ftt-2(n4426)	12.6 ± 0.2	100	-20		<0.001
	hcf-1(-);ftt-2(-) (#1)	18.9 ± 0.4	94	20	50	<0.001
	hcf-1(-);ftt-2(-) (#2)	19.2 ± 0.4	81	22	53	<0.001
#2	N2	15.1 ± 0.2	105			
	hcf-1(pk924)	19.8 ± 0.4	104	31		<0.001
	ftt-2(n4426)	14.2 ± 0.2	100	-6		0.002
	hcf-1(-);ftt-2(-) (#1)	18.8 ± 0.3	88	24	32	<0.001
	hcf-1(-);ftt-2(-) (#2)	19.4 ± 0.4	78	28	37	<0.001

Experiment was carried out at 25°C.
All survival analyses were performed using SPSS software Kaplan Meier analysis and log-rank test to compute *p*-values. *p*-value<0.05 is considered statistically significant.

Kaplan Meier analysis - experiments pooled

	Strain	Mean LS + SEM(Days)	Total N	% lifespan extension vs. N2	% lifespan extension vs. *ftt-2(-)*	*p*-value vs. N2
1+2	N2	15.5 ± 0.1	212			
(Fig 2.5D)	*hcf-1(pk924)*	21.0 ± 0.3	209	36		<0.001
	ftt-2(n4426)	13.4 ± 0.2	200			<0.001
	hcf-1(-);ftt-2(-)	19.1 ± 0.2	341		43	<0.001

Data from two independent experiments and two double mutant isolates are pooled.

into the nucleus and regulates the expression of a large number of genes that together contribute to the diverse functions of IIS, including the regulation of development, metabolism, stress response, and longevity (Lee *et al.*, 2003; McElwee *et al.*, 2003; Murphy *et al.*, 2003). To determine how the *hcf-1*- and *sir-2.1*-responsive DAF-16- target genes compare with the IIS-responsive DAF-16 targets, we further compared the *hcf-1(-)* and *sir-2.*1(O/E) profiles to that of the *daf-2(-)* profile (microarray data from *daf-2(e1370) vs. daf-16(mgDf50);daf-2(e1370)* (Shaw *et al.*, 2007)). Interestingly, expression of the majority of the shared *hcf-1(-)*/*sir-2.1*(O/E)-regulated genes (693/866= 80%) were also changed in *daf-2(-)* in the same direction, yet this represented only a fraction of all *daf-2(-)*-induced changes (693/2515= 28%) (Figure 2.6C,D). This indicates that, among a large number of potential DAF-16 targets, *hcf-1* and *sir-2.1* converge to co-regulate a distinct subset of these genes. Our findings from the microarray comparisons support the model that HCF-1 and SIR-2.1 antagonize each other to control a particular aspect of the DAF-16-regulated transcriptional program.

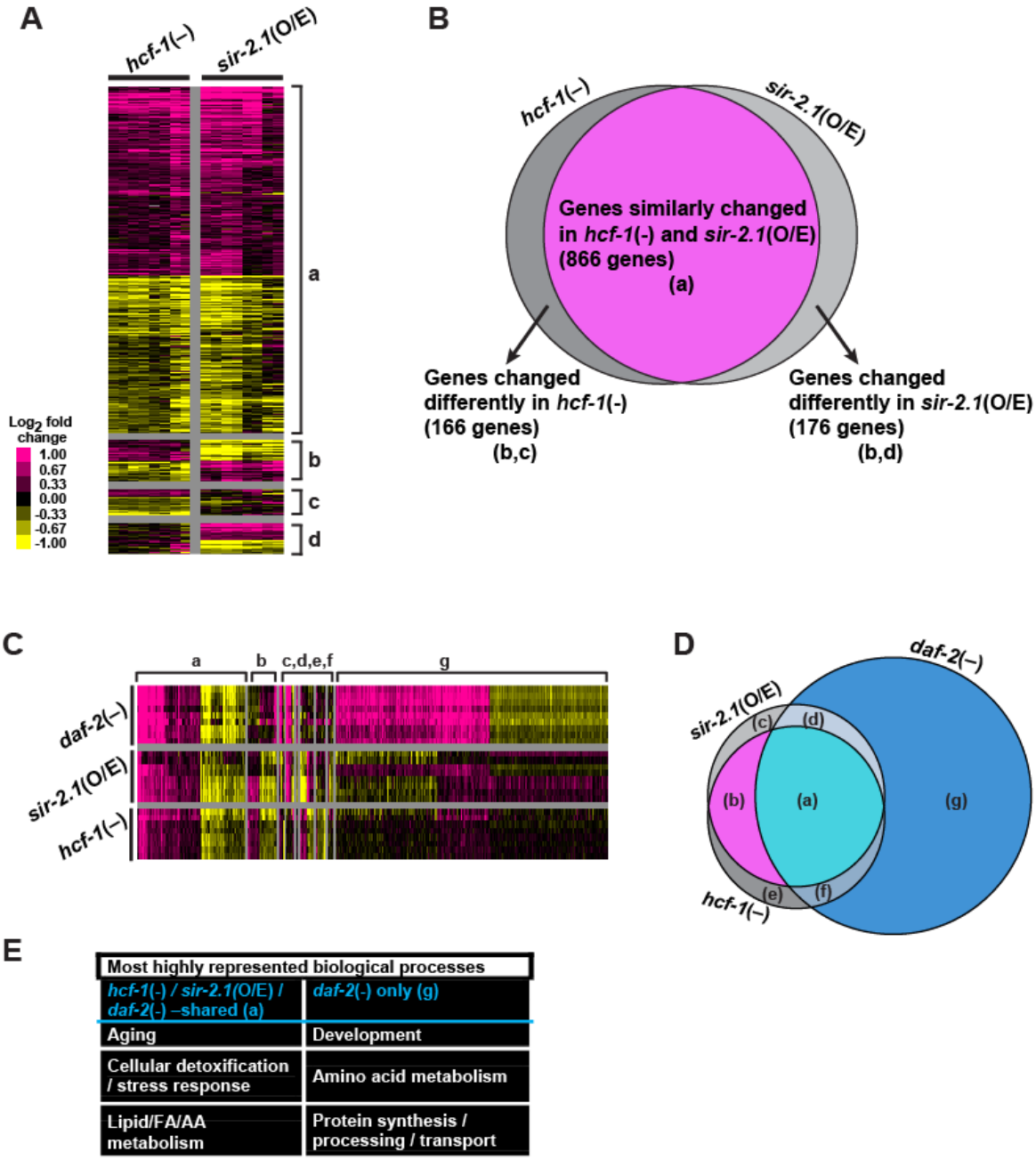
To examine the biological processes that can be carried out by genes affected by *hcf-1(-)* and *sir-2.1*(O/E), we queried their Gene Ontology (GO) terms using Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Dennis *et al.*, 2003). We focused on the GO term categories most significantly enriched in our dataset compared to the *C. elegans* genome. Our analyses revealed that for the DAF-16 target genes co-regulated by HCF-1/SIR-2.1, GO terms for aging, cellular detoxification (in particular phase 1 & 2 detoxification) and stress response were highly overrepresented among both the upregulated and downregulated genes (Figure 2.6E, Table 2.3) (Xu *et al.*, 2005; McElwee *et al.*, 2007). To test whether the DAF-16 targets that are co-regulated by HCF-1/SIR-2.1/DAF-2 might participate in biological functions distinct from the targets uniquely regulated by DAF-2 (and not affected by HCF-1/SIR-2.1), we compared the GO terms represented in the *hcf-1(-)*/*sir-2.*1(O/E)-shared genes to those in *daf-2(-).* Among the genes induced by DAF-16, the most

prominent functional categories represented in the *hcf-1(-)/sir-2.1*(O/E)/*daf-2(-)* overlapping set were very similar to those in the *hcf-1(-)/sir-2.1(*O/E)-co-regulated set (i.e. aging, detoxification, stress response) (Figure 2.6E, Table 2.3). By contrast, the DAF-16 target genes that are uniquely upregulated in *daf-*2*(-)* are enriched for GO categories for developmental, metabolic (amino acid anabolism/catabolism) and cellular ion transport processes (Figure 2.6E, Table 2.3A). Among the genes repressed by DAF-16, the *hcf-1(-)*, *sir-2.*1(O/E) and *daf-2(-)* overlapping set is also enriched with GO terms in aging and stress responses, as well as a new category in fatty acid/lipid/amino acid metabolic processes. Interestingly, the *daf-2(-)*-specific genes are highly enriched for GO terms in protein biosynthesis, protein degradation, unfolded protein response, protein homeostasis, development and cell division (Figure 2.6E, Table 2.3B). Thus, our results suggest that in response to *hcf-1* inactivation and *sir-2.1* overexpression, DAF-16 specifically induces longevity assurance genes to combat toxic cellular insults/stressors and extend lifespan without strongly affecting developmental, and protein homeostasis pathways.

DAF-16 directly binds a consensus DAF-16 binding element (DBE) to regulate the expression of many downstream target genes (Furuyama *et al.*, 2000; Curran *et al.*, 2009). To further investigate how the HCF-1/SIR-2.1-coregulated vs. the IIS-specific DAF-16 target genes might be regulated, we analyzed the 1.5 kb upstream promoter sequences of genes in each group to identify any transcription factor binding sites and regulatory elements that are overrepresented. We submitted the upstream sequences of all genes in *hcf-1/sir-2.1*-coregulated or *daf-2*-specific categories to two *de novo* motif finding algorithms, BioProspector (Liu *et al.*, 2001) and Regulatory Sequence Analysis Tools (RSAT) (Thomas-Chollier *et al.*, 2008) and focused on the top highest-scoring motifs from each algorithm. These analyses revealed four common motifs enriched in the promoters of DAF-16 targets, regardless of their responsiveness to HCF-1 & SIR-2.1 (Table 2.3C), suggesting that DAF-16 likely collaborates with additional yet-to-be identified co-factors in gene regulation.

Figure 2.6. *hcf-1* inactivation and *sir-2.1* overexpression similarly affect a specific subset of *daf-16* downstream target genes.

(A-D) Heat maps representing the expression patterns of differentially expressed genes identified by Significance Analysis of Microarrays (SAM) and Venn diagrams showing the overlap between different datasets. For heat maps, each column represents a biological replicate and each row is a gene. Pink = upregulated, Yellow=downregulated, Black=not changed. (A) Heat maps comparing *hcf-1(-)* and *sir-2.1*(O/E) arrays. Gene clusters are categorized as: (a)=Genes similarly changed in *hcf-1(-)* and *sir-2.1*(O/E) (866), (b)=genes oppositely changed in *hcf-1(-)* and *sir-2.(* O/E) (98), (c)=genes uniquely changed in *hcf-1(-)* (66), (d)=genes uniquely changed in *sir-2.1*(O/E) (73). (B) Venn diagram summarizing overlap in (A). (C) Heat maps comparing *hcf-1(-), sir-2.1*(O/E), and *daf-2(-)* arrays. Genes are clustered as (a)=similarly expressed in all 3 profiles (693), (b)=similar in only *hcf-1(-)* and *sir-2.1(*O/E) (173), (c)=uniquely changed in *sir-2.1(*O/E) (130), (d)=similar in only *hcf-1(-)* and *daf-2(-)* (26), (e)=uniquely changed in *hcf-1(-)* (140), (f)=similar in only *sir-2.1(*O/E) and *daf-2(-)* (46), (g)=uniquely changed in *daf-2(-)* (1750). (D) Venn diagram summarizing overlaps in (C). (E) Most highly enriched GO terms (See also Tables 2.3A,B) are summarized based on general biological process.



E

Most highly represented biological processes	
hcf-1(-) / *sir-2.1*(O/E) / *daf-2*(-) –shared (a)	*daf-2*(-) only (g)
Aging	Development
Cellular detoxification / stress response	Amino acid metabolism
Lipid/FA/AA metabolism	Protein synthesis / processing / transport

Table 2.3A. Enriched GO terms for upregulated genes				
GO terms	***hcf-1*(-) / *sir-2.1*(O/E) - shared**	***hcf-1*(-) / *sir-2.1*(O/E) / *daf-2*(-) - shared**	***daf-2* (-) only**	**Biological process**
Determination of adult lifespan	5.25	5.83		Aging
Glutathione S-transferase	2.93	3.24		Detoxification
Alcohol dehydrogenase	2.35	2.52		Detoxification
Short-chain dehydrogenase	2.20	2.49	2.14	Detoxification
Cytochrome P450	2.10	2.54		Detoxification
Antioxidant/ Oxidoreductase	1.99	2.20	1.73	Detoxification
Cofactor metabolic process	1.96	2.37		
Zinc-finger RING, BBOX	1.85	0.99		
Metal ion binding	1.72	1.19		
Carboxylesterase (type B)	1.60	1.65	1.02	Detoxification
Lysosome	1.52			Detoxification
Endopeptidase inhibitor (cysteine, serpin)	1.44	1.66		Detoxification
G-protein signaling inhibitor	1.10			Signaling
Dauer development		1.06		Development
Phenol metabolic process		1.05		Metabolism
Collagen / cuticle			13.44	
Glycoprotein/ signaling			6.27	Development: pharyngeal, embryonic, neuronal
Neuropeptide signaling			2.63	Development: pharyngeal
EF-hand calcium binding (calmodulin)			2.50	Development: body morphology, locomotion, egglaying
Peptidase/proteolysis			2.32	Metabolism
Cytoskeleton organization			2.12	
UDP-glucuronosyl/UDP-glucosyltransferase			1.99	Detoxification
LIM domain			1.95	Development: muscle, neuron
Patched / hedgehog receptor			1.85	Development : growth, locomotion
Amino acid metabolism (degradation)			1.70	Metabolism
EGF-like domain			1.64	Development: growth
Organismal growth			1.53	
Anion transport			1.48	Cellular homeostasis
Ion homeostasis			1.13	Cellular homeostasis
Glycolysis/Gluconeogenesis			1.12	Metabolism
Sulfur amino acid biosynthesis			1.07	Metabolism
Intermediate filament			1.01	Development: body morphogenesis

Gene ontology term analysis of genes upregulated in *hcf-1*(-)*, sir-2.1(*O/E), and *daf-2*(-). Functionally clustered GO terms are summarized and represented by Enrichment score (ES) (representing how significantly enriched a group of genes within a gene list is over the whole genome: Enrichment score of 1 = p-value $1e^{-1}$. The higher the ES, the more significantly enriched a functional category). Only GO terms with ES>= 1 are shown. *hcf-1*(-)*/sir-2.1(*O/E)-shared= genes "a+b" (Fig 2.6D), *hcf-1*(-)*/sir-2.1(*O/E)/*daf-2*(-)-shared=genes "a" (Fig 2.6D), *daf-2*(-) only =genes in "g" (Fig 2.6D).

Table 2.3B. Enriched GO terms for downregulated genes				
GO terms	***hcf-1*(-) / *sir-2.1*(O/E) - shared**	***hcf-1*(-) / *sir-2.1*(O/E) / *daf-2*(-) - shared**	***daf-2* (-) only**	**Biological process**
Glycoprotein/signaling	3.95	3.76		Cellular signaling
Amino acid biosynthesis	3.14	1.84		Metabolism
Collagen/cuticle	3.00			
SaposinB	2.41			Metabolism: lipid degradation
Acid phosphatase	2.22	1.96		Metabolism: digestion (lysosomal)
Peptidase (serine)	2.18			Metabolism: proteolysis (lysosomal)
Determination of adult lifespan	2.15	2.54		Aging
Lysozyme	1.99	2.24		Immunity
Ribosomal proteins	1.80	2.24	9.96	Translation
Fatty acid biosynthesis	1.60	1.62		Metabolism
Peptidase/proteolysis (C1)	1.51	1.05		Metabolism
UDP-glucuronosyltransferase	1.50	1.76		Detoxification
N-acetyltransferase	1.44			Detoxification
EGF-like domain	1.40			Signaling
C-type lectin	1.31	1.00		Immunity
Carboxylesterase (type B)	1.27			Detoxification
Peptidase/proteolysis (A1)	1.19	1.36		Metabolism
Iron/sulphur/FAD binding	1.18			
FAD-binding	1.15			
Organismal growth (positive)	1.13		14.18	
Peptidase/proteolysis		1.57		
Metal ion transport/binding		1.08		
Larval development			20.93	Development
Hermaphrodite genitalia devel.			7.89	Development
Mitochondrial			6.47	Protein translation/transport
Translation initiation			4.67	Protein biosynthesis
Protein folding/UPR(Chaperone)			4.54	Protein homeostasis
Aminoacyl-tRNA synthetase			4.25	Protein biosynthesis
Protein transport			4.07	Vesicular biogenesis/transport
Endomembrane system			3.59	Potein transport: ER, Golgi, nuclear
Protein targeting/mitochondria			2.99	Protein transport
Organelle lumen			2.94	
WD40 repeat			2.77	
RNA polymerase			2.49	Transcription
Nuclear pore complex			2.42	Nuclear transport
Proteasome core complex			2.33	Protein degradation
Small ribonucleoprotein			2.18	RNA splicing/degradation. Development
Chromatin/Histones			2.18	Chromatin structure/DNA replication
Proteasome component			1.95	Protein degradation
Organismal growth (negative)			1.95	
Golgi apparatus			1.88	Vesicle /transport
Chaperones			1.77	Protein folding
Tetratricopeptide repeat			1.69	Embryonic

				development
RNA metabolism/processing			1.68	RNA Splicing
Ubq-dependent proteolysis			1.58	Protein degradation
Nuclear migration			1.40	Mitosis / cytokinesis
Cell division			1.39	
Ubiquitin-ligase			1.38	Protein degradation
Embryonic development			1.32	
Heat shock protein DnaJ			1.30	UPR, protein homeostasis
Axon/ neuron projection			1.26	Development
RNA-recognition motif			1.24	RNA processing
Cell division/cyclins			1.23	
cis-trans isomerase			1.18	Protein folding/ immunity
Signal peptide processing			1.15	Protein processing / maturation
AAA+ ABC transporter			1.08	Detoxifications
Gene ontology term analysis of genes downregulated in *hcf-1*(-), *sir-2.1(*O/E), and *daf-2*(-).				

Table 2.3 B continued.

Table 2.3C. Enriched promoter regulatory motifs.				
hcf-1(-) / *sir-2.1*(O/E) / *daf-2*(-) – shared genes (693 genes)		*daf-2*(-) – specific genes (1750 genes)		
Motif	**% of genes with motif**	**Motif**	**% of genes with motif**	**Known consensus transcription factor binding site**
	48%		70%	**Mammalian SP1 p-value<0.001**
	45%		66%	**Mammalian Fhl p-value<0.001**
	16%		13%	
	68%		87%	**Mammalian Oct-1 p-value<0.001**
	68%		42%	
	54%	**Not enriched**		**(1) Mammalian GATA (*C. elegans elt-3,5,6)* p-value<0.0001** **(2) Mammalian Evi-1 (*C. elegans egl-43)*, p-value<0.0001**
	10%	**Not enriched**		
Not enriched			10%	
DBE: T(G/A)TTTAC[d]	51%	**DBE: T(G/A)TTTAC[d]**	45%	

a: BioProspector
b: RSAT
c: Two very similar motifs found by BioProspector and RSAT (reverse complements shown)
d:The DAF-16 binding element (DBE) was not among the top overrepresented motifs but the presence of this sequence on the candidate gene promoters was directly searched using RSAT

We are particularly interested in the motifs that are uniquely enriched in the different groups of genes analyzed. The most notable motif highly enriched in the *hcf-1/sir-2.1*/*daf-2*-overlapping group, but not in the *daf-2*-unique group, was the DAF-16-associated element (DAE) (CTTATCA or TGATAAG), previously discovered as a sequence overrepresented in the promoters (Murphy *et al.*, 2003; Curran *et al.*, 2009) of DAF-16-regulated genes and shown to be directly bound by DAF-16 in *in vitro* gel shift assays (Curran *et al.*, 2009) (Table 2.3C). Interestingly, the DAE represents a GATA-factor binding motif that is highly enriched in promoters of genes whose expression show age-dependent changes and whose transcription is controlled by *C. elegans* GATA-factor homologs *elt-3*, *elt-5*, and *elt-6* (Budovskaya *et al.*, 2008). We further compared the expression profiles of *hcf-1(-)* and *sir-2.1(O/E)* to that of aging worms (Budovskaya *et al.*, 2008), and found that 23% of genes that show age-dependent changes were also represented in our *hcf-1/sir-2.1* co-regulated set (*p*-value < 2.2e-16). The large representation of genes that show age-dependent expression changes in the *hcf-1/sir-2.1* group correlates well with our observation that HCF-1 and SIR-2.1 together regulate aging- and stress response-specific DAF-16 downstream targets (Figure 2.6E). Results from the motif analysis also suggest that HCF-1 and SIR-2.1 likely engage additional transcriptional partners, such as GATA factors, in their regulation of DAF-16.

HCF-1 forms a protein complex with SIR-2.1 and 14-3-3 proteins in *C. elegans*

Our genetic and microarray analyses suggest that SIR-2.1 likely antagonizes HCF-1 to regulate DAF-16 activity. To elucidate the molecular mechanism by which SIR-2.1 may inhibit HCF-1, we first tested whether HCF-1 expression or stability is affected by SIR-2.1. We found that the mRNA and protein levels of HCF-1 did not significantly differ in strains lacking or overexpressing *sir-2.1* (data not shown). Since both SIR-2.1 and HCF-1 are known to form a protein complex with DAF-16 in *C. elegans* (Berdichevsky *et al.*, 2006; Li *et al.*, 2008), we next examined whether SIR-2.1 may also physically associate with HCF-1. We

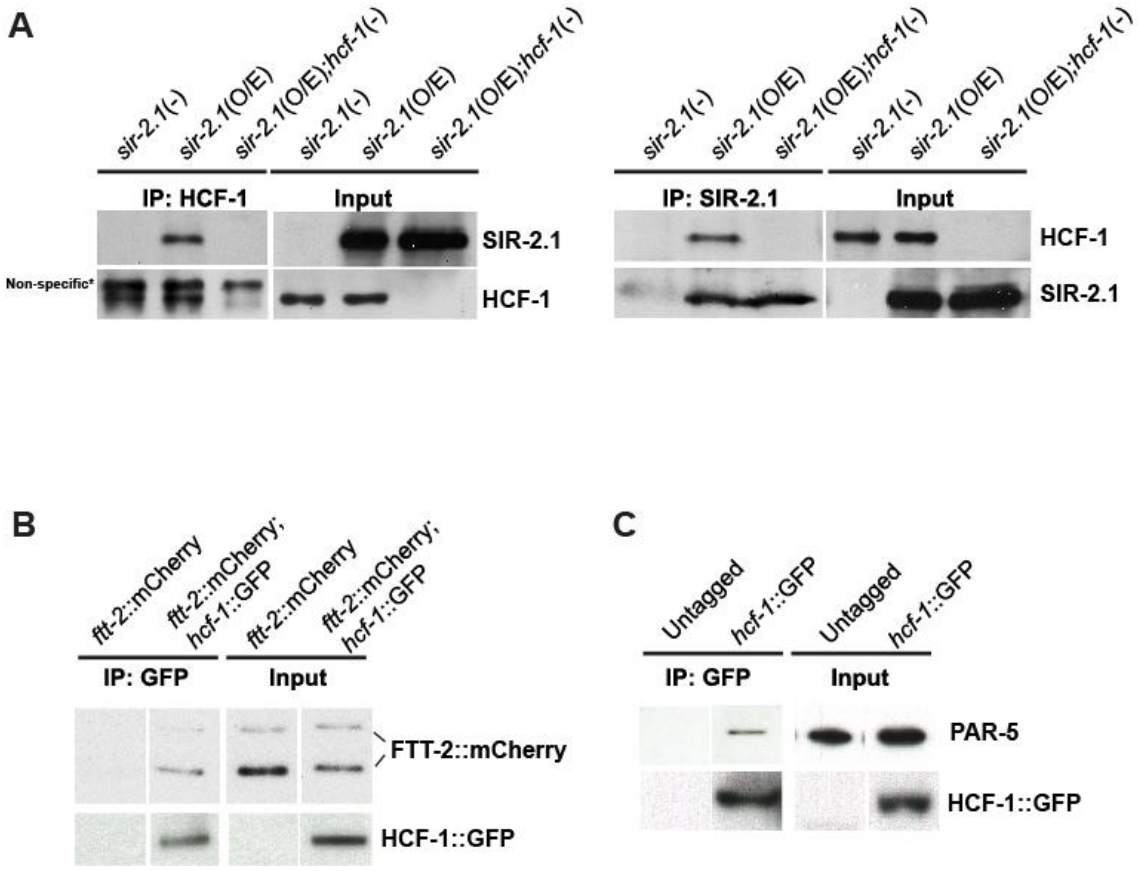
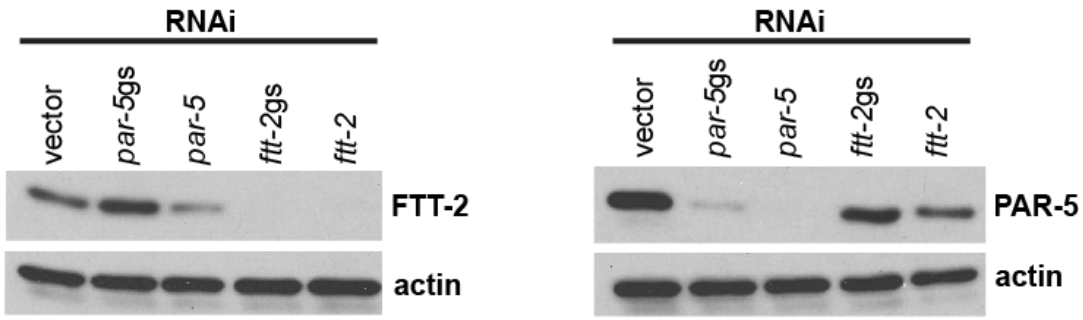


Figure 2.7. *C. elegans* HCF-1 physically interacts with SIR-2.1 and 14-3-3 proteins. (A) Lysates from *sir-2.1*(-) *(sir-2.1(ok434)), sir-2.1*(O/E) *(geIn3[sir-2.1*(O/E)*])*, and *sir-2.1*(O/E)*;hcf-1(-) (hcf-1(ok559);geIn3[sir-2.1*(O/E)*])* worms were either immunoprecipitated using anti-HCF-1 antibody (left panel) or anti-SIR-2.1 antibody (right panel). The immunoprecipitated protein complexes were subsequently immunoblotted using anti-HCF-1, or anti-SIR-2.1 antibodies. (B) Lysates from *ftt-2::mCherry* or *hcf-1::GFP;ftt-2::mCherry* were immunoprecipitated with an anti-GFP antibody and blotted with anti-mCherry or anti-GFP antibodies. (C) Wild-type or HCF-1::GFP expressing worm lysates were immunoprecipitated using anti-GFP antibodies and blotted with anti-PAR-5 or anti-GFP antibody.

A



B

Sequence name	Public name	Peptide coverage	Identified peptides
F52D10.3a	FTT-2a	31.5 %	VTELGAELSNEER, DICQDVLNLLDK, YLAEVASGDDR, SQQSYQEAFDIAK, *QAFDDAIAELDTLNEDSYK*, DSTLIMQLLR**
M117.2	PAR-5	29.0 %	KVTEQGQELSNEER, VTEQGQELSNEER, LLDEFLIVK, YLAEVASEDR, AYQEALDIAK, *QAFDDAIAELDTLNEDSYK*, DSTLIMQLLR**

Figure 2.8. HCF-1 physically interacts with FTT-2 and PAR-5.

(A) *hcf-1(pk924)* worms were grown on plates containing vector control, non-specific (*ftt-2* and *par-5*) or gene-specific (*ftt-2gs* or *par-5gs*) *14-3-3* RNAi until young adult stage and protein levels analyzed by western blotting using anti-FTT-2 or anti-PAR-5 antibodies. Actin was used as a loading control. (B) Sequences of the peptides from FTT-2 and PAR-5 proteins, which were identified in the mass spectrometrical analysis of HCF-1::GFP-bound proteins, are listed. *: these peptides are common to both FTT-2 and PAR-5.

performed co-immunoprecipitation (co-IP) experiments using an affinity-purified anti-HCF-1 antibody and immunoprecipitated HCF-1 from lysates of *geIn3[sir-2.1*(O/E)*]*, worms overexpressing SIR-2.1 to a greater extent than the *pkIs1642[sir-2.1*(O/E)*]* strain we used for lifespan analysis, *hcf-1(pk924);geIn3[sir-2.1*(O/E)*]*, worms overexpressing SIR-2.1 but lacking *hcf-1*, and *sir-2-1(ok434)*, worms lacking *sir-2.1*. SIR-2.1 was co-immunoprecipitated with HCF-1 only in the *geIn3[sir-2.1*(O/E)*]* lysate (Figure 2.7, left panel). A similar complex formation was also detected in reciprocal co-immunoprecipitation experiments (Figure 2.7, right panel).

Since 14-3-3 proteins are proposed to bridge the physical interactions between SIR-2.1 and DAF-16, especially under stress conditions (Berdichevsky *et al.*, 2006; Wang *et al.*, 2006), and our genetic data revealed that 14-3-3 likely function downstream of HCF-1 in longevity modulation, we tested a possible physical association of HCF-1 with 14-3-3 proteins. We immunoprecipitated GFP-fused HCF-1 using anti-GFP antibodies from *hcf-1::gfp;ftt-2::mCherry* or *hcf-1::gfp* strains and blotted with anti-mCherry or anti-PAR-5 antibodies to monitor mCherry-tagged FTT-2 and endogenous PAR-5 respectively. HCF-1 was able to form a protein complex with either FTT-2 or PAR-5 (Figures 2.7B,C). Consistent with the Co-IP results, a search for HCF-1 binding partners using immunoprecipitation of HCF-1::GFP followed by mass spectrometrical analysis of co-purifying proteins identified the two 14-3-3 proteins FTT-2 and PAR-5 (Figure 2.8B). Interestingly, sequence analysis (by scansite.mit.edu) predicts that HCF-1 contains a highly significant consensus 14-3-3 binding site, suggesting HCF-1 may directly bind 14-3-3. Taken together, our data reveal that HCF-1 is a new component in the regulatory network involving SIR-2.1, 14-3-3, and DAF-16.

Acetylation of *C. elegans* HCF-1 is not affected by SIR-2.1 activity

The mammalian homolog of SIR-2.1, SIRT1, is a protein deacetylase and is well known to modulate the activity of target proteins by deacetylation of acetylated lysine

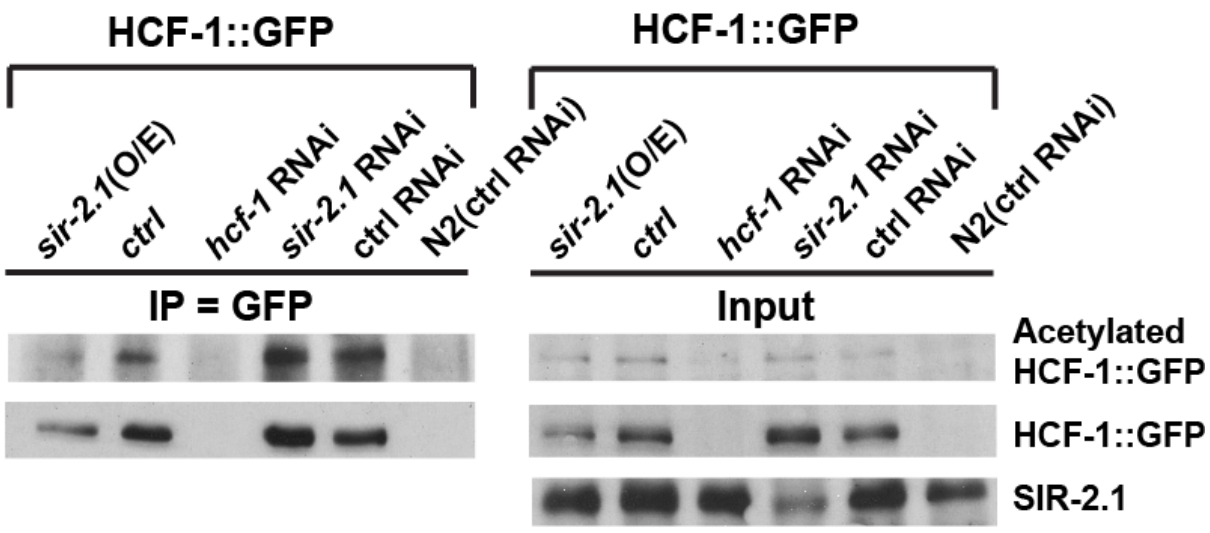


Figure 2.9. Acetylation status of *C. elegans* HCF-1 is not affected by SIR-2.1 levels

Mixed populations of worms of indicated genotypes were either grown on OP50 bacteria (*hcf-1::GFP; pkIS1642 [sir-2.1*(O/E)] vs *hcf-1::GFP*) or fed with vector, *sir-2.1*, or *hcf-1*-targeting RNAi bacteria. HCF-1::GFP was immunoprecipitated from whole worm lysates with an anti-GFP antibody and immunocomplexes analyzed by anti-acetylated lysine antibody to detect acetylated HCF-1. The levels of HCF-1 acetylation were not affected by either *sir-2.1* knockdown or overexpression when compared to the amount of total immunoprecipitated protein in each condition.

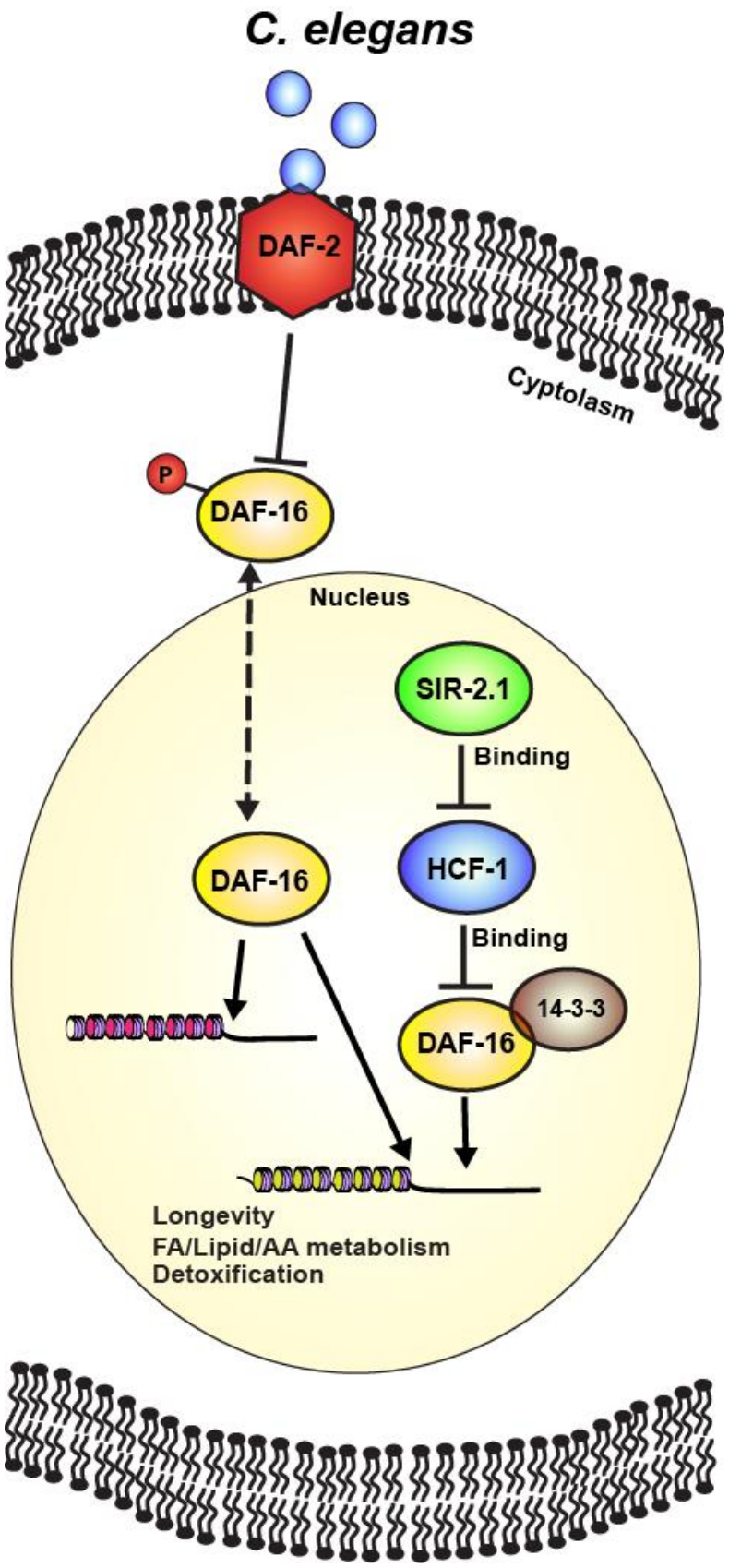
residues, we examined whether SIR-2.1 affects HCF-1's acetylation levels. We used a strain overexpressing GFP-tagged HCF-1 protein, immunoprecipitated HCF-1::GFP from either worms carrying *pkIs1642[sir-2.1*(O/E)*]* or from worms treated with *sir-2.1* RNAi, and probed for acetylation using a pan-acetyl-lysine antibody. We detected acetylated HCF-1 protein in control strains indicating that *C. elegans* HCF-1 is acetylated under basal conditions as has been reported for mammalian HCF-1 (Cai *et al.*, 2010). Yet, neither in *sir-2.1* overexpressing worms not *sir-2.1* depleted animals did we observe a noticeable change in the acetylation levels of HCF-1 (Figure 2.9). Intriguingly, Terri Iwata in our lab showed dramatic increases in mammalian HCF-1 acetylation in cells treated with nicotinamide, a known SIRT1 inhibitor, or shRNA targeting SIRT1 (data not shown), consistent with the idea that SIRT1 in mammals promotes the deacetylation of HCF-1. Our findings indicate that SIR-2.1 likely does not deacetylate HCF-1 in worms and that the regulation of HCF-1 acetylation by SIRT1 could represent an added complexity unique to the mammalian system.

DISCUSSION

The highly conserved FOXO transcription factors are master regulators of diverse biological processes (van der Horst and Burgering, 2007) and as such, their transcriptional activities are tightly controlled (Essers *et al.*, 2005; Berdichevsky *et al.*, 2006; Berman and Kenyon, 2006; Lehtinen *et al.*, 2006; Wolff *et al.*, 2006 ; Li *et al.*, 2008). Although a number of different transcriptional co-factors of DAF-16/FOXO have been identified, little is known about how they functionally interact to fine-tune DAF-16/FOXO activity, and in particular, how they may collaborate to affect DAF-16-mediated lifespan extension. In this study, we identified the DAF-16 nuclear co-repressor HCF-1 as an integral component of the regulatory network involving SIR-2.1/SIRT1, 14-3-3, and DAF-16/FOXO with major consequences to both organismal aging and stress response. Our data indicate that in *C. elegans*, HCF-1 likely functions downstream of SIR-2.1, and upstream of 14-3-3, to regulate a distinct subset of

Figure 2.10. Regulation of DAF-16 by HCF-1 and SIR-2.1.

We propose that *C. elegans* HCF-1 and SIR-2.1 coordinate to fine-tune the transcriptional activity of DAF-16 on a distinct subset of potential target genes. DAF-16 target genes responsive to the *hcf-1/sir-2.1* pathway largely overlap with a small subset of IIS-regulated genes, and are specialized in longevity determination, cellular defense, and lipid/fatty acid/amino acid homeostasis. HCF-1 likely represses DAF-16 by forming a complex with SIR-2.1 and 14-3-3, and antagonizing their abilities to stimulate DAF-16. Our results highlight HCF-1 to be a key component of the regulatory network linking SIR-2.1 and DAF-16 in worms.


C. elegans
DAF-2
Cyptolasm
P
DAF-16
Nucleus
SIR-2.1
Binding
HCF-1
DAF-16
Binding
14-3-3
DAF-16
Longevity
FA/Lipid/AA metabolism
Detoxification

DAF-16 target genes to affect longevity and oxidative stress response. Furthermore, HCF-1 protein participates in protein complex formation with SIR-2.1/SIRT1, 14-3-3, and DAF-16/FOXO in worms (Figure 2.10).

Our expression profiling studies indicate that the set of DAF-16 target genes co-regulated by *sir-2.1*, *hcf-1*, and *daf-2* (area "a" of Figure 2.6D) is enriched for previously identified longevity-associated genes (annotated as "aging" in GO), whereas the IIS-specific targets (area "g" of Figure 2.6D) are not. This is somewhat unexpected as the *hcf-1* mutant and *sir-2.1* overexpressor strains exhibit lifespan extension phenotypes that are much milder than that of the *daf-2* mutant. Interestingly, this correlates well with the degree of expression change observed for many of the shared DAF-16 target genes, as they often exhibit more robust expression changes in the *daf-2(-)* profile compared to the *sir-2.1*(O/E) or *hcf-1(-)* profiles. An implication from this observation is that the co-regulated gene set is particularly important for longevity determination, and may thus contain additional targets important for prolonged lifespan that are not currently known to affect aging.

Our previous genetic findings indicated that reduced insulin signaling synergizes with inactivation of *hcf-1* to affect longevity and DAF-16-mediated gene regulation (Li *et al.*, 2008). We interpreted those results to suggest that IIS and *hcf-1* likely act independently to regulate DAF-16/FOXO. However, a caveat of that interpretation is that the *daf-2* mutant we examined was not a null mutant, and formally, loss of *hcf-1* can further decrease IIS signaling to further increase lifespan. Similarly, the genetic relationship between the insulin signaling pathway and *sir-2.1* has been unclear due to several conflicting reports (Tissenbaum and Guarente, 2001; Berdichevsky *et al.*, 2006). In the current study, a comparison of the DAF-16-regulated gene expression changes in response to either *daf-2* mutation, *hcf-1* inactivation, or *sir-2.1* overexpression indicates that a large majority of the HCF-1/SIR-2.1 co-regulated DAF-16 target genes are similarly regulated by reduced IIS. It is possible that upon downregulation of IIS, the majority of DAF-16 migrates into the nucleus but is still subject to

regulation by nuclear co-factors. Under this scenario, SIR-2.1 and HCF-1 may be acting as additional "gate keepers" to control DAF-16 activation in the face of reduced IIS. In addition, we saw that the insulin/IGF-1-like peptide, *ins-7,* which was shown to act as a *daf-2* agonist (Murphy *et al.*, 2003), was significantly repressed by *hcf-1* inactivation and *sir-2.1* overexpression. Thus, a possible feedback mechanism in which *hcf-1* inactivation or *sir-2.1* activation leads to further inhibition of IIS may also explain the genetic results observed with reduced IIS and *hcf-1* inactivation or *sir-2.1* overexpression.

Our motif analyses revealed additional factors that are likely involved in the regulation of DAF-16 by HCF-1 and SIR-2.1 in *C. elegans*, in particular the aging-related GATA-factor homologs (ELT-3, -5, -6) known to bind the DAE element, a consensus motif enriched in many of the HCF-1/SIR-2.1 co-regulated genes (Budovskaya *et al.*, 2008). Of note, the DAE sequence also shares close resemblance to the mammalian transcription factor Evi1 binding site. Although the *C. elegans* Evi1 homolog, *egl-43*, has been shown to be involved in early development (Rimann and Hajnal, 2007), a function in longevity and stress response has not been reported. Future functional analysis of HCF-1/SIR-2.1 and ELT-3, -5, -6, and EGL-43 will likely yield new insights into additional layers of DAF-16 regulation.

We found that HCF-1 physically associates with DAF-16/FOXO and SIR-2.1/SIRT1 in worms. Previous studies in *C. elegans* indicate that 14-3-3 proteins act as bridging molecules that bring SIR-2.1 and DAF-16 into a protein complex in the nucleus (Berdichevsky *et al.*, 2006; Wang *et al.*, 2006). Interestingly, our data suggest 14-3-3 proteins also physically associate with HCF-1. This raises the question of how these different molecules coordinately interact to affect each other's activities. An intriguing model may be that HCF-1 normally binds 14-3-3/DAF-16 and dampens the ability of DAF-16 to activate its target genes; upon appropriate upstream signals, SIR-2.1 ejects HCF-1 off the complex and induces full activation of DAF-16.

In conclusion, our findings establish a novel link between two evolutionarily

conserved DAF-16/FOXO regulators. This study expands our understanding of the complex role that nuclear factors play in determining the specificity of DAF-16/FOXO activity. These results further implicate HCF-1 as a novel factor that may affect mammalian aging and age-related pathologies through interactions with SIRT1 and FOXO.

MATERIALS AND METHODS

C. elegans strains

All strain stocks were kept at 16°C and grown under standard growth conditions (Brenner, 1974). The strains used are: Wild type N2, *hcf-1(pk924), daf-16(mgDf47);hcf-1(pk924)* (Li *et al.*, 2008), IU372.1 *sir-2.1(ok434)* (7 times outcrossed in our lab), NL3908 *pkIs1641* [*unc-119*], NL3909 *pkIs1642* [*unc-119 sir-2.1*] (Berdichevsky *et al.*, 2006), IU91.1 *pkIs1641* [*unc-119*] (1X outcrossed in our lab), IU94 *pkIs1642* [*unc-119 sir-2.1*](1X outcrossed in our lab), *geIn3[sir-2.1 rol-6(su1006)]* (Tissenbaum and Guarente, 2001) (1X outcrossed in our lab), *ftt-2(n4426)* (Berdichevsky *et al.*, 2006) (3X outcrossed in our lab), *rwIs23* [*hcf-1(pk924);Phcf-1::GFP unc-119],* GR1680 *rwIs23[Phcf-1::GFP; unc-119]; IsB[pCR270(Pftt-2::ftt-2:: Spep-TEV-mCherry::ftt-2-3'UTR; Cb_unc-119)], rwIs9[Phcf-1::hcf-1::GFP Pmec-7::RFP]*. Standard genetic methods were utilized to construct the following strains: *sir-2.1(ok434) hcf-1(pk924), hcf-1(pk924);pkIs1642[sir-2.1*(O/E)*], hcf-1(ok559);geIn3[sir-2.1 rol-6(su1006)], ftt-2(n4426);hcf-1(pk924). daf-16(mgDf50); pkIs1642[sir-2.1*(O/E)*]* was a gift from M. Viswanathan and L. Guarente at MIT (Viswanathan *et al.*, 2005).

Lifespan analysis

All lifespan assays were performed at 25°C unless otherwise noted on Nematode Growth Media (NGM) plates seeded with *E. coli* OP50 or RNAi bacteria. For experiments using OP50, bacteria was grown overnight at 37°C, OD measured after growth and

concentrated to OD 7.5 (5X OP50) or used directly, at OD 1.5 (1X). 35mm NGM plates were seeded with 150uL of OP50 for egglay plates and dried at room temperature. Plates that would be used for transferring worms throughout the lifespan assay were prepared by adding FUDR to OP50 culture to a final concentration of 50ug/mL per plate, seeding 150uL/plate, drying at room temperature, and storing at 4°C until use. For RNAi experiments, HT115 bacteria containing vectors expressing dsRNA were grown at 37°C in LB with 100ug/mL carbenicillin and 15ug/mL tetracycline to OD 0.8, induced with 4mM IPTG for 4 hrs, and either concentrated to OD 7.5 and seeded, or seeded at OD 1.5 (1X). RNAi plates were also induced with 4mM IPTG before use. Well-fed gravid adult worms were allowed to lay eggs at room temperature and the progeny were grown at 25°C until young adult/early gravid adult stage. The synchronized adults were transferred to fresh FUDR-containing plates at Day 0, 2, and 4 of adulthood. For lifespan assays carried out at 20°C, worms were incubated at 25°C for the first three days of adulthood to reduce vulva protrusion defects. The adult worms were scored every other day and worms that did not move when gently prodded by a platinum wire pick were recorded as dead. Worms that bagged, crawled onto the wall of the plate, or had a large protruding vulva were censored on the day of the event. All survival data were analyzed using Kaplan-Meier statistics (SPSS software) to generate statistical values and survival curves. *p*-values were calculated using the log-rank test.

Stress assays

Paraquat: 50-60 synchronized worms were grown on three 60mm NGM/OP50 plates (per strain) at 25°C until day two of adulthood, either directly transferred or washed off the plates with M9 buffer and dispensed into three wells of a 24-well culture plate, and paraquat (Sigma) added to 150mM or 200mM final concentration. Plates were kept covered by aluminum foil to prevent excessive light from degrading paraquat, and rocked on a shaker at 25°C. Survival was scored at the indicated time points after paraquat exposure.

tert-Butyl hydroperoxide: Synchronized worms were grown on OP50 plates until day one of adulthood and transferred onto plates containing 6mM *tert*-Butyl hydroperoxide (*t*-BOOH) (Sigma). Survival was scored at indicated time points after treatment.
Kaplan Meier analysis and Log-rank statistics (SPSS software) were used to generate survival curves, calculate mean survival, and compute statistics. The mean variation in survival of each strain as compared to either wild-type or *pkIs1641[sir-2.1*(wt)*]* was calculated and further analyzed by Linear Mixed model analysis (Breslow and Clayton, 1993) to obtain averaged mean variations relative to control from two or three independent experiments. *hcf-1(-)* and *sir-2.1(-) hcf-1(-)* or *sir-2.1*(O/E) and *hcf-1(-)*;*sir-2.1*(O/E) were entered as "fixed effect" and experiments as "random effect". Linear Mixed model analysis allows statistical evaluation of differences between various treatments (mutants) by taking into account the experimental variation.

RNA isolation and Microarray preparation

For *hcf-1(-)* microarrays, total RNA was purified from synchronized L4 or young adult(YA) worms. Worms were synchronized by allowing hypochlorite-treated eggs to hatch in M9 buffer for 20 hrs at 16°C, and plating 500 L1 stage worms onto each of 5-6 10mm NGM plates seeded with 3X OP50 bacteria. 6 biological replicates of *hcf-1(-)/daf-16(-);hcf-1(-),* two replicates of *hcf-1(-)/*N2 were prepared. The synchronized populations were grown to L4 or YA stage at 25°C and harvested by washing off the plates with M9 buffer and freezing the worm pellet in liquid nitrogen. Total RNA was isolated using Tri-reagent (Molecular Research Center, Inc.) (Troemel *et al.*, 2006) and purified with the RNeasy kit (Qiagen). cRNA synthesis/amplification, Cy3/Cy5 dye labeling, and hybridization onto Agilent 4X44K *C. elegans* oligonucleotide microarrays were performed as previously described (Shaw *et al.*, 2007). Half the arrays were dye-flip replicates in each comparison.

Details on *sir-2.1*(O/E) microarrays will be published elsewhere (Rogers*, Jan*,

Ashraf, and Murphy, in preparation). *daf-2(-)* microarray data were published in (Shaw *et al.*, 2007).

Microarray analysis

hcf-1(-) microarrays: Hybridized microarray slides were washed according to Agilent instructions , and images were scanned using an Axon Instruments GenePix 4000B scanner, reading at wavelengths of 635nm and 532nm (Axon Instruments, http://www.axon.com) (Pleiss *et al.*, 2007). The arrays were scanned at three different PMT settings to capture spots with low and high signal, and later combined to create a single dataset. The image data were uploaded onto the Princeton University MicroArray database (PUMA [http://puma.princeton.edu]). Log_2 transformed fold change data were acquired after normalizing, filtering for array and spot quality, collapsing replicate spots to a mean value on PUMA.

Data for *sir-2.1*(O/E) and *daf-2(-)* arrays were similarly normalized and processed on PUMA.

SAM analysis: Log_2 transformed fold change data with no cutoff were submitted to SAM (Tusher *et al.*, 2001). One class analysis was used to identify genes significantly and consistently changed in each database. Two-class unpaired analysis was used to identify genes similarly and divergently changed between different datasets. Genes found to be significantly changed at 0% FDR in only *hcf-1(-), sir-2.1(*O/E), or *daf-2(-)* using one-class analysis, and similarly and divergently changed between different datasets using two-class unpaired analysis were combined and sorted based on the SAM output to generate heat maps using Treeview (Eisen *et al.*, 1998).

Gene Ontology classification: Worm Base IDs (WBID) of genes identified in SAM were pasted into the Functional annotation clustering tool in DAVID (http://david.abcc.ncifcrf.gov/) for gene annotation enrichment analysis (Dennis *et al.*, 2003; Huang da *et al.*, 2009). Functional annotation clustering was performed with the default

criteria and enrichment score for each annotation cluster was determined.

Upstream regulatory motif analysis: 1.5 kb upstream sequences were submitted to BioProspector (http://ai.stanford.edu/~xsliu/BioProspector/) (Liu *et al.*, 2001) and RSAT (http://rsat.ulb.ac.be/rsat/) (Thomas-Chollier *et al.*, 2008) to identify overrepresented cis-regulatory elements. An oligonucleotide length of 8 bp was specified for both algorithms. The highest scoring (most significantly enriched) 10 motifs from BioProspector and 5 motifs from RSAT were obtained. As BioProspector returns the same sequences multiple times, only unique motifs were reported. Motifs were displayed in WebLogo (weblogo.berkeley.edu) (Crooks *et al.*, 2004). The matrices associated with each motif were submitted to the TomTom motif comparison tool (http://meme.sdsc.edu/meme/cgi-bin/tomtom.cgi) (Gupta *et al.*, 2007) to compare against a database of known transcription factor binding sites (Transfac).

Immunoprecipitation and Mass spectrometry

Immunoprecipitation was performed as described (Li *et al.*, 2008). For HCF-1/SIR-2.1 co-IPs, mixed stage worms were grown on plates, harvested, and sonicated in IP lysis buffer (50 mM HEPES pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% Glycerol, 0.1% Triton X-100, 1 mM sodium fluoride, 2.5mM sodium orthovanadate, 1mM PMSF, and Complete (EDTA-free) protease inhibitor cocktail) and lysates cleared by centrifugation. Lysates were incubated with either affinity purified guinea-pig anti-HCF-1 antibody (Li *et al.*, 2008) or rabbit anti-SIR-2.1 antibody (Novus Biologicals) at 4°C overnight. Immunocomplexes were incubated with trysacryl protein A-agarose beads (Pierce) at 4°C for four hours, washed four times with IP lysis buffer, and eluted by boiling in SDS sample buffer. Eluted protein complexes were analyzed by western blotting using the anti-HCF-1, anti-SIR-2.1, or anti-actin (Chemicon, clone C4) antibodies. For acetylation experiments, HCF-1 was immunoprecipitated from the HCF-1::GFP overexpressing strains using a mouse monoclonal

anti-GFP antibody (Invitrogen) and analyzed by western blotting with anti-acetylated Lysine (Cell signaling), anti-GFP(Clontech), and anti-SIR-2.1 antibodies.

For Mass spectrometry and 14-3-3 co-IPs, GFP-tagged HCF-1 was purified from mixed stage *C. elegans*, using a previously reported method (Cheeseman *et al.*, 2004) with slight modifications. In short, worms were grown in liquid culture as mixed stages to a density of 4000 worms/mL. Worms were washed into lysis buffer (50 mM HEPES at pH 7.4, 1 mM EGTA, 1 mM MgCl2, 150 mM KCl, 10 % (v/v) glycerol, protease and phosphatase inhibitors), drop-frozen in liquid nitrogen, and ground using a mortar and pestle. Resulting powder was thawed and NP-40 was added to 0.05 % (v/v). Immunoprecipitations were conducted on a 20,000 g supernatant of this extract, using monoclonal mouse-α-GFP antibody (Invitrogen) coupled to Protein A resin (Biorad). Immunoprecipitated proteins were eluted using 100 mM glycine at pH 2.6. For co-IPs, eluted protein complexes were analyzed by western blotting using anti-mCherry (Ruvkun Lab, MGH Boston) or rabbit anti-PAR-5 (a kind gift from K.J. Kemphues, Cornell University) antibodies. For mass-spectrometrical analysis, immunoprecipitated proteins were eluted using 100 mM glycine at pH 2.6. Eluted proteins were visualized by silver-stained SDS-PAGE and identified by mass spectrometry. For the latter, samples were digested using trypsin and the resulting peptides were separated via nano-capillary liquid chromatography and identified by online tandem mass spectrometry (LTQ-XL, Thermo). Mass spectra were searched against the current wormpep database using Sequest (Thermo) and DTASelect (Tabb *et al.*, 2002).

As a negative-control for the mass-spectrometrical analysis, an identical purification was conducted using *C. elegans* expressing only untagged endogenous HCF-1. IP and negative-control were compared using Contrast (Tabb *et al.*, 2002).

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CHAPTER 3

HOST CELL FACTOR 1 INHIBITS SKN-1 TO MODULATE OXIDATIVE STRESS RESPONSES IN *C. ELEGANS*[2]

ABSTRACT

Host Cell Factor-1 (HCF-1) is a conserved longevity determinant traditionally involved in transcriptional regulatory network formation and regulation of gene expression in diverse biological processes. *C. elegans* HCF-1 is a key nuclear repressor of DAF-16-mediated transcription influencing lifespan and stress responses. SKN-1 transcription factor in *C. elegans* is an evolutionarily conserved protector against oxidative and xenobiotic stress and is a well-established pro-longevity factor. Here we demonstrate that SKN-1 contributes to the enhanced oxidative stress resistance incurred by *hcf-1* inactivation in a manner parallel to DAF-16. This functional interaction between HCF-1 and SKN-1 specifically occurs under excessive oxidant stress as SKN-1 is dispensable for the thermotolerance and long lifespan produced by *hcf-1* deficiency. Analogous to its impact on DAF-16-mediated transcription, HCF-1 represses the transcriptional activation of SKN-1 to inhibit SKN-1 target genes such as *gcs-1* and multiple glutathione S-transferases involved in cellular detoxification pathways. To control SKN-1 activity, HCF-1 prevents nuclear accumulation of SKN-1 in response to oxidative stress. Our findings reveal a novel and context-specific regulatory relationship between two highly conserved longevity and stress response factors HCF-1 and SKN-1.

[2] This chapter will be summarized as a manuscript. I conceived all the experiments, performed all stress assays (except Fig 3.1A), N2/*hcf-1(-)* microarrays, RT-qPCR experiments, and analyzed all the data. Colette Picard assisted with microarray analyses, sample preparation for RT-qPCR, and performed lifespan experiments, *skn-1(zu67);gcs-1::gfp* experiment, and SKN-1::GFP nuclear translocation experiments. Charles Pereyra performed the paraquat stress assay shown in Figure 3.1A.

INTRODUCTION

Animal cells are equipped with mechanisms to cope with fluctuations in the Reactive Oygen Species (ROS) produced as by-products of oxygen metabolism. However, excessive elevation of environmental as well as intracellular free radicals may lead to disruption of redox homeostasis and contribute to the development of various human diseases including cancer (Reuter *et al.*, 2010), diabetes (Rains and Jain, 2011), cardiovascular disease (Molavi and Mehta, 2004), and neurodegenerative disorders (Jomova *et al.*, 2010). When eukaryotic organisms encounter oxidative stress, several major signaling cascades and transcription factors respond by mounting the expression of a battery of detoxifying and stress response enzymes to defend against the harmful effects of oxidation. Two strikingly conserved mechanisms responsible from combating oxidative stress involve FOXO forkhead and NF-E2-related (Nrf) transcription factors. FOXO proteins are activated upon encountering elevated free radical stress and counteract ROS by increasing the expression of several antioxidant enzymes such as superoxide dismutase and catalase (Honda and Honda, 1999; Murphy *et al.*, 2003; van der Horst and Burgering, 2007). On the other hand, Nrf1 and Nrf2 transcription factors initiate a Phase II detoxification response via inducing the expression of free radical scavenger and cellular repair enzymes comprising γ-glutamyl cysteine synthetase (GCS-1), glutathione S-transferases (GSTs), and UDP-glucuronosyl transferases (UGTs). The highly conserved nature of oxidative stress defense pathways allow for detailed exploration of the regulatory mechanisms governing the activation of these pathways in model organisms such as *Caenorhabditis elegans.*

C. elegans SKN-1 is the sole ortholog of Nrf factors and similarly orchestrates a robust oxidative stress response (An and Blackwell, 2003). Under non-stressed conditions, the majority of SKN-1 protein is retained in the cytoplasm in an inactive form and thus its transcriptional activity is minimal. When worms are challenged by increased oxidative stress, SKN-1 enters the nuclei of intestinal cells where it activates a battery of select target genes to

mount an insult-specific detoxification response (An and Blackwell, 2003; Oliveira *et al.*, 2009). Therefore, disruption of *skn-1* function confers hypersensitivity to oxidative stress (An and Blackwell, 2003). To ensure proper and timely activation of SKN-1, its activity is regulated by several different mechanisms. SKN-1 activation is governed at the level of subcellular distribution and protein stability. While inhibitory phosphorylation by Glycogen Synthase Kinase-3 (GSK-3) and insulin/IGF-1 like signaling cascade (IIS) sequesters SKN-1 in the cytoplasm to prevent its constitutive activation, phosphorylation by the p38 MAP Kinase pathway in response to stress promotes accumulation of SKN-1 in intestinal nuclei (An *et al.*, 2005; Inoue *et al.*, 2005; Tullet *et al.*, 2008). SKN-1 is also regulated by ubiquitin-mediated degradation by the action of core proteasomal components (Kahn *et al.*, 2008; Choe *et al.*, 2009). Besides its fundamental role in coping with stress, SKN-1 is an integral player in several different longevity pathways in worms. The prolonged lifespan conferred by reductions in IIS, translation inhibition, and dietary restriction is mediated in part by SKN-1 (Bishop and Guarente, 2007; Tullet *et al.*, 2008; Wang *et al.*, 2010). Although it is established that SKN-1 is a transcription factor capable of integrating versatile upstream stimuli to modulate stress responses and longevity, a comprehensive understanding of how its activation is fine-tuned and what additional regulators affect its transcriptional activity is lacking.

Host cell factor-1 (HCF-1) is an evolutionarily conserved transcriptional regulator involved in key biological processes such as cell cycle regulation, aging, and stress responses (Lee *et al.*, 2007; Tyagi *et al.*, 2007; Li *et al.*, 2008). In mammals, the main function of HCF-1 is to gather various transcriptional regulatory complexes to regulate gene expression (Wysocka and Herr, 2003). In *C. elegans*, HCF-1 represses the major longevity determinant DAF-16, the worm ortholog of FOXO transcription factors (Li *et al.*, 2008). *hcf-1* deficiency results in lifespan extension and heightened oxidative and heavy metal stress tolerance in a manner dependent on *daf-16* (Li *et al.*, 2008). HCF-1 protein predominantly resides in the

nucleus, physically associates with DAF-16 and in doing so sequesters DAF-16 away from a subset of its target gene promoters thus inhibiting DAF-16-mediated transcription (Li *et al.*, 2008). The high level of conservation between mammalian and *C .elegans* HCF factors implies that HCF-1 likely engages in numerous transcriptional regulatory networks to carry out its functions. Yet, the exact mechanism by which HCF-1 influences lifespan and stress responses and the involvement of additional HCF-1 interactors have not been fully elucidated.

We tested whether HCF-1 functionally interacts with SKN-1 to modulate longevity and stress responses in worms. We illustrate here that SKN-1 mediates the oxidative stress resistance of *hcf-1* mutants but is not required for the long lifespan and moderate heat stress tolerance of *hcf-1* deficient worms. The basal expression of a subset of SKN-1 target genes such as *gcs-1* and *gst*s important for Phase II detoxification are induced in the absence of *hcf-1*. We further show that HCF-1 prevents nuclear accumulation of SKN-1 under oxidative stress conditions. Taken together, our data indicate that HCF-1 inhibits the transactivation of SKN-1 in response to oxidative stress by limiting SKN-1's nuclear accumulation.

RESULTS

skn-1 mediates the oxidative stress resistance but not the thermotolerance or lifespan extension conferred by *hcf-1(pk924)* mutation

Loss of function of *hcf-1* has been shown to increase resistance to oxidative stress induced by paraquat exposure. This elevated paraquat-resistance of *hcf-1* mutant worms is partially dependent on *daf-16* (Li *et al.*, 2008). Whether *hcf-1* engages only *daf-16* or any additional factors to regulate the response to oxidative insults is not established. Given that *skn-1* is a major mediator of detoxification following oxidative stress in *C. elegans* (An and Blackwell, 2003), we assessed if *skn-1* participates in the regulation of oxidative stress response by *hcf-1*. We diminished *skn-1* activity by treating *hcf-1(pk924)* and wild-type N2 worms with RNAi targeting *skn-1* (Oliveira *et al.*, 2009), and measured the survival of worms

exposed to paraquat. As previously observed (Li *et al.*, 2008), *hcf-1* mutants treated with control RNAi survived on paraquat much longer than did N2 worms (Figure 3.1A, 3.2A; Table 3.1A, 3.2A). Knocking down *daf-16* conferred sensitivity to paraquat and partially suppressed the improved endurance of *hcf-1* mutants (Figures 3.1A, 3.2A; Tables 3.1A, 3.2A). Interestingly, *skn-1* was also necessary for the resistance of *hcf-1(pk924)* since reducing *skn-1* activity blunted the paraquat-response exhibited by *hcf-1* mutants (Figures 3.1A,3.2A; Tables 3.1A, 3.2A). Therefore, our data suggest that HCF-1 inhibits both DAF-16 and SKN-1 during elevated oxidative stress caused by paraquat exposure.

Besides paraquat, SKN-1 mounts defenses against the toxic effects of various other compounds including *tert*-butyl hydroperoxide (*t*-BOOH), an oxidizing agent, and Sodium Arsenite (NaAs), a heavy metal (An *et al.*, 2005; Inoue *et al.*, 2005; Oliveira *et al.*, 2009), and is activated under thermal stress (An and Blackwell, 2003). *hcf-1* was previously shown to regulate a response to heavy-metal-induced toxicity (Li *et al.*, 2008). We wondered whether *skn-1* is specifically important for the paraquat-induced oxidative stress response by *hcf-1* or is a general mediator of stress response downstream of *hcf-1*. We first examined the survival of *hcf-1(pk924)* mutants treated with *t*-BOOH or NaAs or subjected to heat shock at 32°C. We found that *hcf-1(pk924)* worms displayed elevated resistance to all (Figure 3.1B, Figures 3.2B-D; Table 3.1B, Tables 3.2B-D). Inactivating *daf-16* attenuated the heightened *t*-BOOH, NaAs resistance and thermotolerance of *hcf-1* mutants, whereas *skn-1* knockdown sensitized *hcf-1* mutants to *t*-BOOH and NaAs but not heat shock (Figure 3.1B, Figures 3.2B-D; Table 3.1B, Tables 3.2B-D). Interestingly, depleting *skn-1* from *hcf-1* mutants resulted in a moderate increase in thermotolerance, suggesting that a different factor may be overactivated in the combined absence of *skn-1* and *hcf-1*. These observations illustrate that HCF-1 engages SKN-1 under different conditions that generate oxidative stress but work independently of SKN-1 under thermal stress.

Both *hcf-1* and *skn-1* are implicated as prominent longevity modulators. Absence of

Figure 3.1. Both *skn-1* and *daf-16* are necessary for the oxidative stress resistance conferred by *hcf-1(pk924)* mutation.

(A) *skn-1* and *daf-16* deficiency suppresses the paraquat stress tolerance of *hcf-1* mutant worms. N2 wild-type or *hcf-1(pk924)* worms were grown at 25°C on plates with corresponding control (L4440) or gene-targeting RNAi until Day one of adulthood and transferred to plates containing 25mM paraquat. Survival was scored once a day. The results were reproduced in three independent experiments. One representative experiment is shown. (B) *skn-1* and *daf-16* are required for the increased survival of *hcf-1* mutant worms exposed to *t*-BOOH. Following an overnight egglay at 16°C, worms were allowed to develop at 25°C and exposed to 4mM *t*-BOOH on plates. Survival was monitored every 4-10 hours. *daf-16* RNAi was used once whereas results with *skn-1* knockdown were repeated three times. (C) *skn-1* does not mediate the long lifespan of *hcf-1* deficient animals. After an overnight egglay at 16°C, worms were incubated at 25°C for the remainder of the lifespan assay and number of dead worms scored every two days. Pooled data from two independent experiments for *daf-16* RNAi and three experiments for ctrl and *skn-1* RNAi are combined and plotted. (D) Further diminishing *skn-1* levels in an RNAi sensitive *rrf-3* mutant strain does not shorten *hcf-1* mutant lifespan. Experiment was carried out as in (C). Data from one experiment for *daf-16* RNAi and two independent experiments for the rest are displayed.
Refer to Tables 3.1A-D for quantitative analyses.

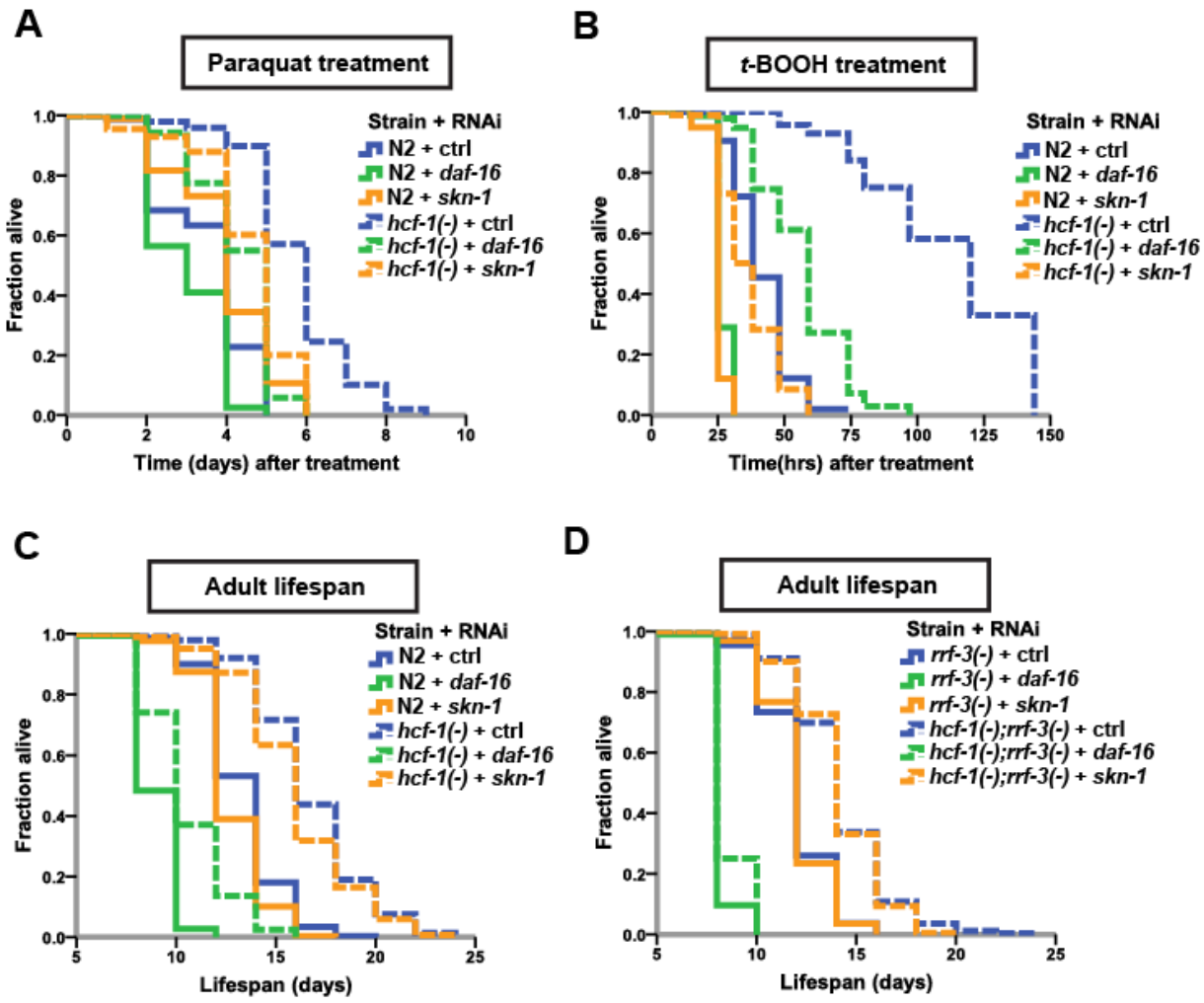

A
Paraquat treatment
Strain + RNAi
N2 + ctrl
N2 + daf-16
N2 + skn-1
hcf-1(-) + ctrl
hcf-1(-) + daf-16
hcf-1(-) + skn-1
Fraction alive
Time (days) after treatment
B
t-BOOH treatment
Strain + RNAi
N2 + ctrl
N2 + daf-16
N2 + skn-1
hcf-1(-) + ctrl
hcf-1(-) + daf-16
hcf-1(-) + skn-1
Fraction alive
Time(hrs) after treatment
C
Adult lifespan
Strain + RNAi
N2 + ctrl
N2 + daf-16
N2 + skn-1
hcf-1(-) + ctrl
hcf-1(-) + daf-16
hcf-1(-) + skn-1
Fraction alive
Lifespan (days)
D
Adult lifespan
Strain + RNAi
rrf-3(-) + ctrl
rrf-3(-) + daf-16
rrf-3(-) + skn-1
hcf-1(-);rrf-3(-) + ctrl
hcf-1(-);rrf-3(-) + daf-16
hcf-1(-);rrf-3(-) + skn-1
Fraction alive
Lifespan (days)

Table 3.1A

Epistasis between *hcf-1(pk924)* and *skn-1* RNAi in paraquat

Strain + RNAi	Mean Survival + SEM(Days)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. *hcf-1(pk924)* + ctrl	% effect on N2 + ctrl	% effect by *hcf-1(pk924)* vs. corresponding N2+RNAi
N2 + ctrl	3.5 ± 0.2	85		<0.001		
N2 + *daf-16*	3.0 ± 0.2	92	0.072	<0.001	-15	
N2 + *skn-1*	4.0 ± 0.2	91	0.012	<0.001	13	
hcf-1(pk924) + ctrl	5.8 ± 0.2	74	<0.001		64	64
hcf-1(pk924) + *daf-16*	4.3 ± 0.1	84	<0.001	<0.001	23	44
hcf-1(pk924) + *skn-1*	4.6 ± 0.2	66	<0.001	<0.001	29	15

L4440 RNAi construct was used as ctrl RNAi. One representative experiment out of three independent repeats is shown. Worms were incubated at 25°C from egglay to the end of the experiment. Similar results were obtained at 20°C. RNAi was initiated at egglay. Day 1 adult worms were transferred to plates containing 25mM paraquat and survival was scored daily.
All survival analyses were done using SPSS software, Kaplan Meier analysis and log-rank test to compute *p*-values. *p*-value <0.05 is considered statistically significant.

Table 3.1B

Epistasis between *hcf-1(pk924)* and *skn-1* RNAi in *t*-BOOH

Strain + RNAi	Mean Survival + SEM(Hrs)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. *hcf-1(pk924)* + ctrl	% effect on N2 + ctrl	% effect by *hcf-1(pk924)* vs. corresponding N2+RNAi
N2 + ctrl	41.7 ± 1.1	105		<0.001		
N2 + *daf-16*	26.6 ± 0.4	76	<0.001	<0.001	-36	
N2 + *skn-1*	25.0 ± 0.3	100	<0.001	<0.001	-40	
hcf-1(pk924) + ctrl	108.0 ± 2.1	95	<0.001		159	159
hcf-1(pk924) + *daf-16*	56.5 ± 1.7	96	<0.001	<0.001	36	112
hcf-1(pk924) + *skn-1*	36.5 ± 1.1	104	0.002	<0.001	-12	45

Experiment was carried out once with *daf-16* RNAi. Worms were transferred to 25°C following an overnight egglay at 16°C and kept at 25°C for the remainder of the experiment. RNAi was initiated at egglay. Day 1 adult worms were transferred to plates containing 4mM *t*-BOOH and survival was scored multiple times during the day.

Table 3.1C

Epistasis between *hcf-1(pk924)* and *skn-1* RNAi in lifespan

Strain + RNAi	Mean Survival + SEM(Days)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. *hcf-1(pk924)* + ctrl	% effect on N2 + ctrl	% effect by *hcf-1(pk924)* vs. corresponding N2+RNAi
N2 + ctrl	13.2 ± 0.1	302		<0.001		
N2 + *daf-16*	9.0 ± 0.1	189	<0.001	<0.001	-32	
N2 + *skn-1*	12.7 ± 0.1	299	<0.001	<0.001	-4	
hcf-1(pk924) + ctrl	16.7 ± 0.2	295	<0.001		26	26
hcf-1(pk924) + *daf-16*	10.5 ± 0.2	294	<0.001	<0.001	-20	17
hcf-1(pk924) + *skn-1*	16.0 ± 0.1	310	<0.001	0.014	21	26

Pooled data from two independent experiments for *daf-16* RNAi and three experiments for ctrl and *skn-1* RNAi are displayed. Worms were transferred to 25°C following an overnight egglay at 16°C and kept at 25°C for the remainder of the experiment. RNAi was initiated at egglay. Worms were transferred to fresh RNAi plates at Day 0 (Young adult), D2, D4, and D8 of adulthood.

Table 3.1D

Epistasis between *hcf-1(pk924)* and *skn-1* RNAi in lifespan in *rrf-3* RNAi sensitive background

Strain + RNAi	Mean Survival + SEM(Hrs)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. *hcf-1(pk924)* + ctrl	% effect on N2 + ctrl	% effect by *hcf-1(pk924)* vs. correspondi ng N2+RNAi
rrf-3(pk1426) + ctrl	12.0 ± 0.1	197		<0.001		
rrf-3(pk1426) + *daf-16*	8.2 ± 0.1	98	<0.001	<0.001	-32	
rrf-3(pk1426) + *skn-1*	12.0 ± 0.1	199	0.970	<0.001	0	
rrf-3(pk1426);hcf-1(pk924) + ctrl	14.2 ± 0.2	261	<0.001		19	19
rrf-3(pk1426);hcf-1(pk924) + *daf-16*	8.5 ± 0.1	101	<0.001	<0.001	-29	4
rrf-3(pk1426);hcf-1(pk924) + *skn-1*	14.1 ± 0.1	240	0.002	0.570	18	17

Data from one experiment for *daf-16* RNAi and pooled data from two independent experiments for ctrl and *skn-1* RNAi are displayed. Worms were transferred to 25°C following an overnight egglay at 16°C and kept at 25°C for the remainder of the experiment. RNAi was initiated at egglay. Worms were transferred to fresh RNAi plates at Day 0 (Young adult), D2, D4, and D8 of adulthood.

hcf-1 prolongs lifespan in a *daf-16*-dependent manner (Li *et al.*, 2008). On the other hand, expression of *skn-1* in the ASI head neurons is required for the lifespan extension induced by liquid Dietary Restriction (lDR) (Bishop and Guarente, 2007) and its expression in the intestine contributes to the longevity of IIS mutants (Tullet *et al.*, 2008). Considering the roles of *hcf-1* and *skn-1* in longevity determination and our observations suggesting that *hcf-1* regulates *skn-1* activity under oxidative stress conditions prompted us to further investigate the functional relationship between *hcf-1* and *skn-1* in lifespan. We measured the lifespan of wild-type and *hcf-1(pk924)* mutant worms fed with control, *daf-16,* or *skn-1* RNAi. As expected, *daf-16* knockdown substantially attenuated the long-lifespan of worms harboring the *hcf-1* mutation (Figure 3.1C; Table 3.1C). On the contrary, reducing *skn-1* levels did not significantly alter the longevity associated with *hcf-1* mutation (Figure 3.1C; Table 3.1C). To rule out the possibility that *skn-1* inactivation did not have an effect on *hcf-1* mutant lifespan due to inefficient RNAi knockdown, we utilized the RNAi-hypersensitive mutant *rrf-3(pk1426)* (Simmer *et al.*, 2002). Corroborating the results of experiments conducted in the wild-type background, depleting *skn-1* in the *rrf-3(-);hcf-1(-)* double mutant did not curtail the prolonged lifespan of this mutant whereas depleting *daf-16* did (Figure 3.1D; Table 3.1D). We were unable to generate strains carrying both the *hcf-1* and *skn-1* mutations since these two genes are closely linked on the same chromosome and the *skn-1* mutation needs to be balanced (www.wormbase.org). Taken together, our findings indicate that while DAF-16 contributes to all phenotypes associated with *hcf-1* mutation, SKN-1 is specifically regulated by HCF-1 to modulate oxidative stress response but is dispensable for thermal stress and lifespan functions of HCF-1.

skn-1 and *daf-16* act in parallel downstream of *hcf-1* to respond to oxidative stress

Our results, which demonstrate that HCF-1 inhibits both DAF-16 and SKN-1 under oxidative stress conditions, raised the question of whether DAF-16 and SKN-1 act in concert

or independently downstream of HCF-1. Previous work has postulated that SKN-1 and DAF-16 likely operate independently of each other downstream of IIS to regulate the expression of distinct subsets of target genes and modulate longevity (Tullet *et al.*, 2008). Yet this proposal is contradicted by the observation that *daf-16* RNAi could fully suppress the lifespan extension conferred by extra copies of wild-type SKN-1 (Tullet *et al.*, 2008). Therefore, the exact relationship between DAF-16 and SKN-1 functions is incompletely understood. To gain insight into the mechanism with which HCF-1 regulates oxidative stress response, we sought to determine the genetic relationship between DAF-16 and SKN-1 downstream of HCF-1. We fed N2, *hcf-1(pk924), daf-16(mgDf47)* and *daf-16(mgDf47);hcf-1(pk924)* worms control or *skn-1* RNAi, and monitored their survival after exposure to paraquat, *t*-BOOH, and NaAs. Upon treatment with any of the three compounds, simultaneously depleting both *daf-16* and *skn-1* annulled the *hcf-1* mutant-induced resistance to a greater extent than did depleting either one alone (Figures 3.2A-C; Tables 3.2A-C), suggesting that DAF-16 and SKN-1 are activated in *hcf-1* mutants possibly through independent mechanisms and collectively provide protection against oxidative insults. As would be expected, *skn-1* knockdown did not lead to additional hypersensitivity to thermal stress in the absence of *daf-16* corroborating our proposal that DAF-16 but not SKN-1 is engaged by HCF-1 at higher temperatures (Figure 3.2D). Overall, our data are consistent with a model in which HCF-1 inhibits DAF-16 and SKN-1 activities through independent means when worms are challenged by elevated oxidative-stress.

HCF-1 inhibits the expression of SKN-1 target genes important for oxidative stress response and cellular detoxification

SKN-1 activates or represses a variety of downstream target genes by directly associating with a consensus binding site G/ATCAT at the proximal promoter region

Figure 3.2. *skn-1* contributes to the heightened oxidative stress but not heat stress resistance exhibited by *hcf-1(pk924)* mutation in a *daf-16* independent manner.

Survival plots of indicated strains fed with control (L4440) or *skn-1* RNAi. All graphs represent combined data from two independent experiments. In all cases, egglay was allowed to continue overnight at 16°C, after which worms were transferred to 25°C until Day one of adulthood. Animals were subsequently transferred to plates containing (A) 50mM paraquat, (B) 4mM *t*-BOOH, (C) 10mM NaAs or (D) directly transferred to a 32°C incubator. Worms were scored as dead or alive (A) once a day, (B) at 4-10 hour intervals, (C) at 2-4 hour intervals, (D) every 12 hours.

Refer to Tables 3.2A-D for detailed quantitative data.

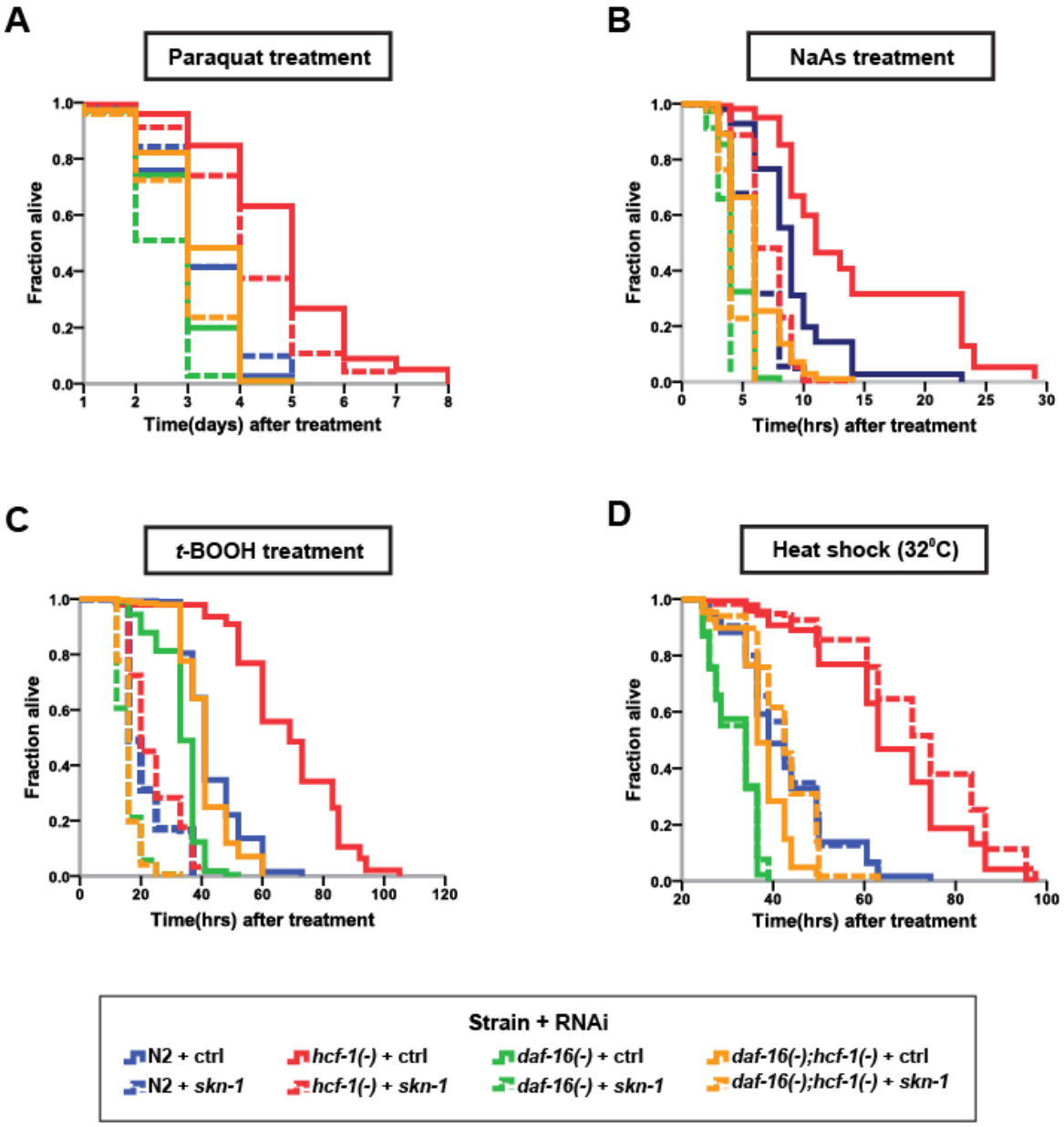

A
Paraquat treatment
Fraction alive
Time(days) after treatment
B
NaAs treatment
Fraction alive
Time(hrs) after treatment
C
t-BOOH treatment
Fraction alive
Time(hrs) after treatment
D
Heat shock (32°C)
Fraction alive
Time(hrs) after treatment
Strain + RNAi
N2 + ctrl
N2 + skn-1
hcf-1(-) + ctrl
hcf-1(-) + skn-1
daf-16(-) + ctrl
daf-16(-) + skn-1
daf-16(-);hcf-1(-) + ctrl
daf-16(-);hcf-1(-) + skn-1

Table 3.2A

Epistasis between *hcf-1(pk924)*, *daf-16(mgDf47)* and *skn-1* RNAi in paraquat

Strain + RNAi	Mean Survival + SEM(Days)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. ctrl RNAi	% effect on N2 + ctrl	% effect on ctrl RNAi
N2 + ctrl	3.2 ± 0.1	230				
N2 + *skn-1*	3.3 ± 0.1	223	0.065	0.065	5	5
hcf-1(pk924) + ctrl	4.8 ± 0.1	218	<0.001		52	
hcf-1(pk924) + *skn-1*	4.2 ± 0.1	217	<0.001	<0.001	30	-14
daf-16(mgDf47) + ctrl	2.9 ± 0.1	210	<0.001		-8	
daf-16(mgDf47) + *skn-1*	2.5 ± 0.0	222	<0.001	<0.001	-22	-14
daf-16(-);hcf-1(-) + ctrl	3.3 ± 0.6	213	0.229		3	
daf-16(-);hcf-1(-) + *skn-1*	2.9 ± 0.1	218	0.001	<0.001	-9	-11

Pooled data from two independent experiments are shown. Worms were transferred to 25°C following an overnight egglay at 16°C and kept at 25°C for the remainder of the experiment. RNAi was initiated at egglay. Worms were transferred to plates containing 50mM paraquat at Day 1 of adulthood and scored daily for survival.

Table 3.2B

Epistasis between *hcf-1(pk924)*, *daf-16(mgDf47)* and *skn-1* RNAi in *t*-BOOH

Strain + RNAi	Mean Survival + SEM(Hrs)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. ctrl RNAi	% effect on N2 + ctrl	% effect on ctrl RNAi
N2 + ctrl	43.3 ± 0.7	230				
N2 + *skn-1*	21.5 ± 0.5	223	<0.001	<0.001	-50	-50
hcf-1(pk924) + ctrl	68.7 ± 1.3	218	<0.001		59	
hcf-1(pk924) + *skn-1*	24.2 ± 0.6	217	<0.001	<0.001	-44	-65
daf-16(mgDf47) + ctrl	33.2 ± 0.5	210	<0.001		-23	
daf-16(mgDf47) + *skn-1*	15.6 ± 0.3	222	<0.001	<0.001	-64	-53
daf-16(-);hcf-1(-) + ctrl	41.1 ± 0.6	210	<0.001		-5	
daf-16(-);hcf-1(-) + *skn-1*	16.2 ± 0.2	222	<0.001	<0.001	-63	-61

Pooled data from two independent experiments are shown. Worms were transferred to 25°C following an overnight egglay at 16°C and kept at 25°C for the remainder of the experiment. RNAi was initiated at egglay. Worms were transferred to plates containing 4mM *t*-BOOH at Day 1 of adulthood and scored every 4-10 hours for survival. .

Table 3.2C

Epistasis between *hcf-1(pk924), daf-16(mgDf47)* and *skn-1* RNAi in NaAs

Strain + RNAi	Mean Survival + SEM(hrs)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. ctrl RNAi	% effect on N2 + ctrl	% effect on ctrl RNAi
N2 + ctrl	9.1 ± 0.2	217				
N2 + *skn-1*	6.0 ± 0.1	214	<0.001	<0.001	-34	-34
hcf-1(pk924) + ctrl	14.6 ± 0.5	222	<0.001		60	
hcf-1(pk924) + skn-1	7.0 ± 0.1	230	<0.001	<0.001	-23	-52
daf-16(mgDf47) + ctrl	4.5 ± 0.1	219	<0.001		-51	
daf-16(mgDf47) + skn-1	3.6 ± 0.0	182	<0.001	<0.001	-60	-21
daf-16(-);hcf-1(-) + ctrl	6.0 ± 0.1	221	<0.001		-34	
daf-16(-);hcf-1(-) + skn-1	4.2 ± 0.1	254	<0.001	<0.001	-54	-30

Pooled data from two independent experiments are shown. Worms were transferred to 25°C following an overnight egglay at 16°C and kept at 25°C for the remainder of the experiment. RNAi was initiated at egglay. Worms were transferred to plates containing 10mM Sodium Arsenite at Day 2 of adulthood and scored every 2-6 hours for survival.

Table 3.2D

Epistasis between *hcf-1(pk924), daf-16(mgDf47)* and *skn-1* RNAi in Heat Shock

Strain + RNAi	Mean Survival + SEM(Hrs)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. ctrl RNAi	% effect on N2 + ctrl	% effect on ctrl RNAi
N2 + ctrl	42.6 ± 0.7	238				
N2 + *skn-1*	43.1 ± 0.7	221	0.705	0.705	1	1
hcf-1(pk924) + ctrl	65.6 ± 1.0	217	<0.001		54	
hcf-1(pk924) + skn-1	72.0 ± 1.1	229	<0.001	<0.001	69	10
daf-16(mgDf47) + ctrl	31.7 ± 0.3	231	<0.001		-26	
daf-16(mgDf47) + skn-1	31.6 ± 0.3	237	<0.001	0.628	-26	0
daf-16(-);hcf-1(-) + ctrl	37.8 ± 0.4	237	<0.001		-11	
daf-16(-);hcf-1(-) + skn-1	42.5 ± 0.5	234	0.444	<0.001	0	12

Pooled data from two independent experiments are shown. Worms were transferred to 25°C following an overnight egglay at 16°C and kept at 25°C until Day 1 of adulthood and switched to 32°C for the remainder of the experiment. RNAi was initiated at egglay. Animals were scored twice a day for survival.

(Blackwell *et al.*, 1994; An and Blackwell, 2003; Oliveira *et al.*, 2009). Given that HCF-1 is a prominent coregulator of transcription factors , we asked whether it affects the transcriptional activity of SKN-1. We hypothesized that if HCF-1 restricts SKN-1's transcriptional activation, reducing HCF-1 function should induce SKN-1-mediated gene expression. To examine the effects of HCF-1 on SKN-1 activity, we monitored the expression of multiple well-studied direct targets of SKN-1 in the absence of *hcf-1*. Under elevated stress or reduced IIS conditions, SKN-1 directly induces detoxification genes such as γ-glutamyl cysteine synthetase (*gcs-1*) and glutathione S-transferases (*gst*) in the intestine (An and Blackwell, 2003; Tullet *et al.*, 2008). We exploited transgenic GFP reporters overexpressing *gcs-1, gst-4,* and *gst-7* (Link *et al.*, 1999; An and Blackwell, 2003; Kahn *et al.*, 2008; Tullet *et al.*, 2008) to monitor SKN-1 activity. These reporters allow for discerning gene expression changes in different tissues of the organism. When we diminished *hcf-1* levels by RNAi knockdown, intestinal expression of all three SKN-1 target genes were elevated (Figure 3.3A). The inflation in gene expression was due to SKN-1 activity and not the activation of another transcription factor or artifactual effects of *hcf-1* inactivation, since the induction was abolished in the *gcs-1::gfp* transgenic worms carrying the *skn-1(zu67)* mutation (Figure 3.3B). These data indicate that HCF-1 normally represses SKN-1-mediated gene expression.

To gain a more global insight into the transcriptional regulation of SKN-1 by HCF-1, we compared the transcriptomes of *hcf-1* mutants to those of *skn-1*-depleted worms. We reasoned that if HCF-1 modulates SKN-1-mediated transcription, there should be at least a partial overlap between HCF-1-regulated and SKN-1-regulated gene expression profiles. To identify genes whose expression was altered in *hcf-1* mutants, we compared the expression profiles of *hcf-1(pk924)* mutant worms to that of N2 wild-type worms (referred to as *hcf-1(-)* profile). We then compared this dataset to previously published gene expression microarrays which identified basally-regulated *skn-1* target genes through comparisons of control versus

Figure 3.3. HCF-1 regulates the transcriptional activity of SKN-1.

(A) Levels of SKN-1 target genes *gcs-1::gfp, gst-4::gfp,* and *gst-7::gfp* are intestinally induced upon *hcf-1* RNAi. Induction of *gcs-1::gfp* in response to IIS kinase *sgk-1* is shown as a control (Tullet *et al.*, 2008). Representative data from multiple independent experiments is displayed. GFP fluorescence of young adult worms was scored blindly as described in (An and Blackwell, 2003; Tullet *et al.*, 2008). Worms were kept on RNAi for two generations, where the first generation was cultured at 16°C and the second generation at 25°C. Number of worms (N): *gcs-1::gfp*+ctrl = 74, *gcs-1::gfp+sgk-1* RNAi = 71, *gcs-1::gfp+hcf-1* RNAi = 89, *gst-4::gfp*+ctrl = 415, *gst-4::gfp+hcf-1* RNAi = 533, *gst-7::gfp*+ctrl = 379, *gst-7::gfp+hcf-1* RNAi = 385. Chi squared test was employed to compute *p*-values where * denotes *p*-value < 0.001. (B) Induction of *gcs-1::gfp* in response to *hcf-1* knockdown is *skn-1*-dependent. The response of *gcs-1::gfp* to *hcf-1* attenuation in the *skn-1(zu67)* mutant is measured. Experiment was carried out as in (A) and done once. N: *gcs-1::gfp*+ctrl = 104, *gcs-1::gfp+hcf-1* = 97, *skn-1(zu67);gcs-1::gfp*+ctrl= 33, *skn-1(zu67);gcs-1::gfp+hcf-1* = 26. (C) Comparison of gene expression profiles of *hcf-1* mutant and *skn-1* RNAi treated worms. Filtered data from two independent microarrays of *hcf-1(pk924)* vs. N2 (denoted *hcf-1(-))* and 7 microarrays of N2+*gfp* RNAi vs. N2+*skn-1* RNAi (denoted *skn-1(+)*) were submitted to Significance analysis of microarrays (SAM) to identify similarly and divergently regulated genes, which were clustered and displayed using Treeview. Yellow represents gene induction and blue represents repression. Refer to Table 3.3 for a summary of enriched Gene Ontology terms associated with indicated clusters. (D) *hcf-1* deficiency induces SKN-1 target genes. Worms were grown on RNAi bacteria from embryo to young adult stage. mRNA levels of indicated genes were quantified by RT-qPCR. All measurements were normalized to actin levels and are displayed as fold change relative to N2+ctrl (L4440) RNAi. Data are averaged from three independent experiments and error bars represent +/- SEM. *p*-values are computed using Student's T-test and $p<0.05$ is considered statistically significant.

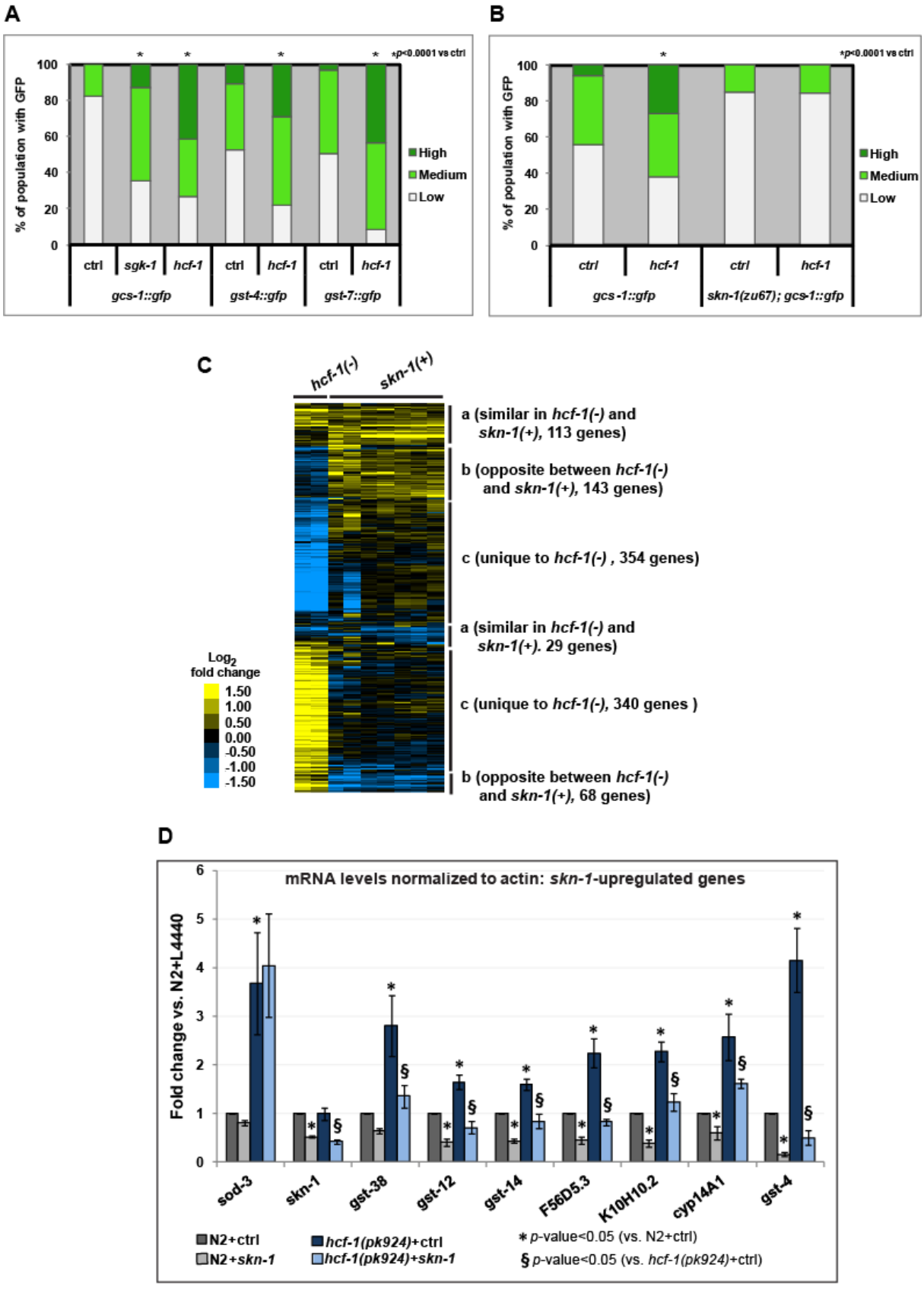

A
B
C
D
% of population with GFP
High
Medium
Low
*p<0.0001 vs ctrl
ctrl
sgk-1
hcf-1
gcs-1::gfp
gst-4::gfp
gst-7::gfp
skn-1(zu67); gcs-1::gfp
hcf-1(-)
skn-1(+)
a (similar in hcf-1(-) and skn-1(+), 113 genes)
b (opposite between hcf-1(-) and skn-1(+), 143 genes)
c (unique to hcf-1(-) , 354 genes)
a (similar in hcf-1(-) and skn-1(+). 29 genes)
c (unique to hcf-1(-), 340 genes)
b (opposite between hcf-1(-) and skn-1(+), 68 genes)
Log2 fold change
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
mRNA levels normalized to actin: skn-1-upregulated genes
Fold change vs. N2+L4440
sod-3
skn-1
gst-38
gst-12
gst-14
F56D5.3
K10H10.2
cyp14A1
gst-4
N2+ctrl
N2+skn-1
hcf-1(pk924)+ctrl
hcf-1(pk924)+skn-1
* p-value<0.05 (vs. N2+ctrl)
§ p-value<0.05 (vs. hcf-1(pk924)+ctrl)

Table 3.3. Enriched GO terms of *hcf-1(-)* and *skn-1(+)* clusters.

DAVID was utilized to determine Gene Ontology terms most significantly overrepresented in *hcf-1(-)/skn-1(+)* overlapping and distinct gene sets. Enrichment Score (ES) is calculated based on how significantly enriched a functional group of genes are compared to their representation in the entire *C. elegans* genome. *p*-value = 10^{-ES}. The higher the ES, the more significantly enriched a biological category.

Table 3.3. Gene Ontology term classification of *hcf-1(-)* and *skn-1(+)* overlapping and non-overlapping genes		
Cluster a (similar)		
GO term	**ES**	**General process**
Glutathione S-transferase	10	Phase II Detoxification
Peptidases	2.6	Proteolysis
Short chain dehydrogenase/reductase	2.2	Phase I Detoxification
Vitamin B6 binding	1.2	Metabolism
Peptidoglycan Metabolic process	3.5	Metabolism
Peptidases	1	Proteolysis
Cluster b (opposite)		
GO term	**ES**	**General process**
Peptidases	3	Proteolysis/Lysosome
Glycoprotein	2.38	Signaling
Pyridoxal phosphate	2.31	Metabolism
Amino acid catabolism	2.28	Metabolism
Multicellular organismal aging	2.27	Aging
NADH binding	1.9	Metabolism
CHK kinase	1.6	Small molecule signaling
Fatty acid biosynthesis	1.49	Metabolism
Lipid modification	1.02	Metabolism
Lectin	1	Immunity
Cluster c (*hcf-1* only)		
GO term	**ES**	**General process**
Cuticle collagen	3.77	Structural component
Major sperm protein	2.96	Reproduction
Carboxylesterase type B	2.23	Lipid metabolism
Multicellular organismal aging	2.17	Aging
Drug metabolism	2.06	Detoxification
Cytochrome P450	1.74	Detoxification
Glycoprotein	1.26	Signaling
Lipid transporter	1.25	Lipid metabolism
EGF-like domain	1.23	Signaling
MATH, BTB/POZ domain	1.2	Transcriptional regulation
Acyl-CoA oxidase/dehydrogenase	1.11	Fatty acid metabolism
FBOX protein	1.09	Ubiquitin-mediated degradation

skn-1 RNAi-treated worms (referred to as *skn-1(+)* profile) (Oliveira *et al.*, 2009). Indeed, we found a small group of genes, the majority of which were upregulated in the absence of *hcf-1* and the presence of *skn-1*, consistent with the idea that SKN-1 activation in *hcf-1* mutants may account for the altered expression of these genes (Cluster a in Figure 3.3B). We selected a number of *hcf-1(-)/skn-1(+)*-upregulated genes and performed Reverse-transcription coupled quantitative PCR (RT-qPCR) to inspect whether the *hcf-1* mutant-induced elevation of these genes was dependent on SKN-1 activity. Consistent with the microarray data, when *skn-1* was knocked down in N2 worms, the mRNA levels of the candidate genes were lessened (Figure 3.3D). Similarly, diminished *skn-1* activity largely abolished the elevated gene expression produced by *hcf-1* mutation (Figure 3.3D). To obtain a more in-depth understanding of what biological processes are represented by the *hcf-1(-)* and *skn-1(+)*-overlapping gene set, we determined the most overrepresented Gene Ontology (GO) terms associated with this group. In support of the idea that HCF-1 specifically affects SKN-1's oxidative stress response functions, we found that among the upregulated genes Phase II detoxification genes such as *gst*s were by far the most highly enriched class. In addition, Phase I detoxification genes including short chain dehydrogenases were highly overrepresented (Table 3.3, Cluster a). Interestingly, categories representing proteolysis and metabolic functions were also observed (Table 3.3). When we expanded our analysis to find GO terms associated with genes oppositely regulated in *hcf-1(-)* vs. *skn-1(+)* and genes uniquely affected in *hcf-1(-)*, we found that lipid/fatty acid/ amino acid metabolism, cellular detoxification, aging, signaling, reproduction, transcriptional and ubiquitin regulation were among the most highly enriched classes (Table 3.3). Our findings establish HCF-1 as a specific transcriptional regulator of SKN-1, where HCF-1 governs SKN-1 activity at a group of genes main function of which is to combat oxidative insults and toxicity.

HCF-1 prevents nuclear accumulation of SKN-1

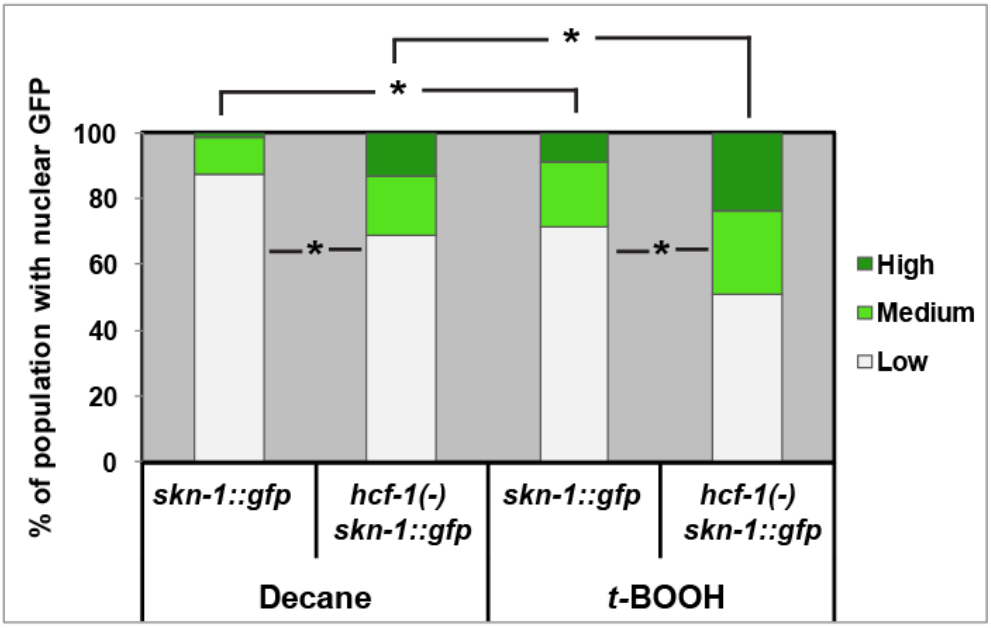
Due to elevated levels of oxidants, reduced activity of *gsk-3* kinase, IIS kinases, or proteasome complex components, or increased activity of the p38 MAPK pathway, SKN-1 accumulates in the nucleus to initiate transcription of target genes (An and Blackwell, 2003; An *et al.*, 2005; Inoue *et al.*, 2005; Kahn *et al.*, 2008; Tullet *et al.*, 2008; Choe *et al.*, 2009). In an effort to understand the mechanism whereby HCF-1 inhibits SKN-1 activity, we asked whether HCF-1 affects the subcellular localization of SKN-1. To test this, we monitored the subcellular distribution of SKN-1 in the presence or absence of *hcf-1*. We used the *hcf-1(pk924)* mutant and monitored its effects on SKN-1's subcellular localization using a transgenic strain carrying the SKN-1B/C::GFP reporter (An and Blackwell, 2003). Under unstressed conditions, attenuating *hcf-1* function increased the nuclear accumulation of SKN-1 protein (Figure 3.4). Considering our genetic data which suggests that SKN-1 is likely overactivated in *hcf-1* mutants upon exposure to oxidative insults, we assessed whether HCF-1 regulates the nuclear accumulation of SKN-1 in worms treated with *t*-BOOH or NaAs. As expected, we observed significantly higher levels of SKN-1::GFP in intestinal nuclei upon exposure to *t*-BOOH and NaAs (Figure 3.4). Interestingly, the amount of nuclear SKN-1 was even further augmented in *hcf-1* mutants (Figure 3.4). Our results indicate that HCF-1 regulates the activity of SKN-1 by inhibiting its nuclear accumulation.

DISCUSSION

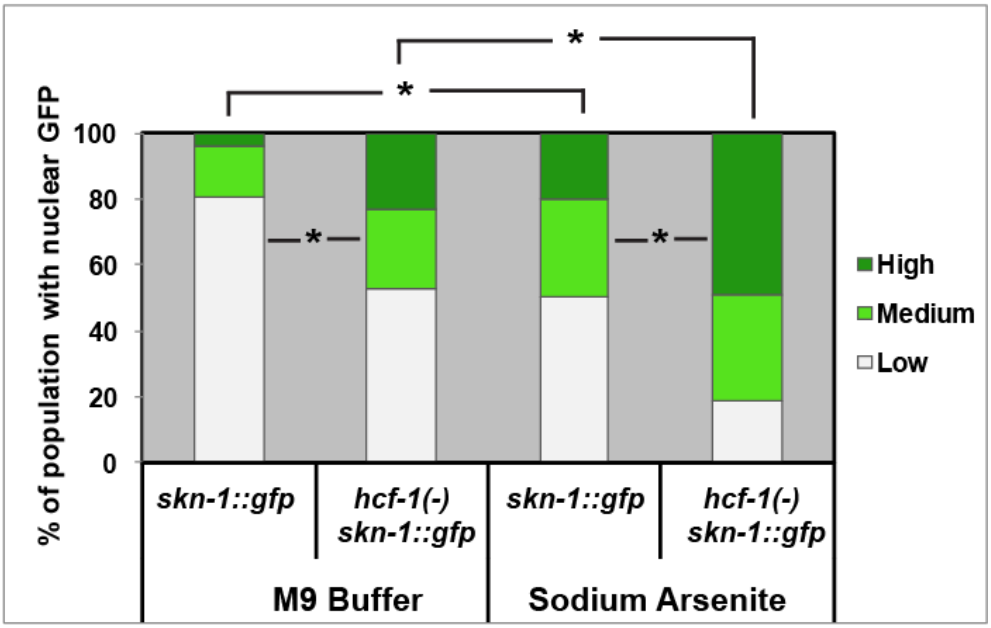
HCF-1 is a versatile transcriptional regulator whose functions in longevity modulation and stress response pathways are just beginning to be elucidated (Li *et al.*, 2008). Although HCF-1 is shown to be a major transcriptional repressor of DAF-16, the mechanistic insights into the molecular actions of HCF-1 have not been fully described. Given that mammalian HCF-1 acts in concert with diverse transcription factors and regulators to achieve transcriptional specificity (Wysocka *et al.*, 2003; Tyagi *et al.*, 2007), and the structural and functional conservation between worm and mammalian HCF proteins, it is anticipated that *C.*

Figure 3.4. SKN-1 accumulation in the nucleus is increased in the absence of *hcf-1*. Wild type or *hcf-1(pk924)* mutant worms transgenically expressing a SKN-1B/C::GFP reporter were grown at 25°C on NGM plates containing OP50 bacteria from L1 until L4 stage, transferred to plates containing (A) *t*-BOOH or decane (as vehicle control) or (B) 5mM NaAs or M9 (as vehicle control), and nuclear SKN-1 levels examined as described in (An and Blackwell, 2003; Tullet *et al.*, 2008) and Materials and Methods. Pooled data from three independent experiments are displayed. * denotes a p-value<0.001. Chi Squared test was used to compute p-valued.

A


% of population with nuclear GFP
100
80
60
40
20
0
*
*
*
*
High
Medium
Low
skn-1::gfp
hcf-1(-) skn-1::gfp
skn-1::gfp
hcf-1(-) skn-1::gfp
Decane
t-BOOH

B


% of population with nuclear GFP
100
80
60
40
20
0
*
*
*
*
High
Medium
Low
skn-1::gfp
hcf-1(-) skn-1::gfp
skn-1::gfp
hcf-1(-) skn-1::gfp
M9 Buffer
Sodium Arsenite

elegans HCF-1 engages multiple partners to accomplish its effects on longevity and stress responses. In this study, we investigated the functional interactions between HCF-1 and a major longevity and stress response regulator SKN-1. We have illustrated that HCF-1 inhibits transcriptional activation by SKN-1 to regulate oxidative stress response but not heat shock response or longevity. Our data indicate that SKN-1 contributes to the elevated oxidative stress resistance conferred by *hcf-1* mutation through a mechanism independent of that carried out by DAF-16 (Figure 3.5). HCF-1 inhibits the transcriptional activation of select SKN-1 target genes by preventing the nuclear accumulation of SKN-1 likely in response to oxidative challenges. Our findings signify a novel mechanism whereby SKN-1 activity is governed by HCF-1.

While HCF-1 requires DAF-16 for all of its longevity and stress-related functions, our data indicate that it interacts with SKN-1 in addition to DAF-16 only under oxidative stress conditions. It is unclear how HCF-1 discriminates between different signals to employ SKN-1 only during a specific scenario. HCF-1 is found in a protein complex with DAF-16, where it prevents promoter localization of DAF-16 (Li *et al.*, 2008). Whether HCF-1 also binds SKN-1 has yet to be determined. Interestingly, SKN-1 carries a conserved HCF-1-binding motif (HBM), DHSY, which is a major site of interaction between HCF-1 and HCF-1-interacting factors in mammalian cells (Freiman and Herr, 1997). It is possible that certain post-translational modifications, for example phosphorylation on specific residues, on HCF-1, SKN-1, or both brought about by elevated oxidation stress may allow HCF-1 to recognize and bind SKN-1via the HBM thereby inhibiting SKN-1. Whether HCF-1 represses SKN-1 activity by sequestering it away from target gene promoters or by recruiting repressive undoubtedly shed light onto the molecular mechanism carried out by HCF-1 to regulate SKN-1 activity.

Our experiments looking at SKN-1's nuclear localization suggest that HCF-1 affects

the nuclear redistribution of SKN-1 under both basal conditions and upon challenging the worms by *t*-BOOH or NaAs treatments, in which case intestinal SKN-1 accumulation is exacerbated in *hcf-1* mutants. This observation contradicts our lifespan epistasis experiments indicating that HCF-1 likely does not engage SKN-1 under basal conditions. However, our gene expression studies indicate that *hcf-1* inactivation is sufficient to induce SKN-1 target genes even under normal culturing conditions. One way to explain these observations is that SKN-1 is only important for HCF-1 to respond to internal or external oxidative stressors and that the ability to cope with oxidative stress may not be a major contributing factor to the longevity of *hcf-1* mutant worms.

Our observation that SKN-1's stress-induced nuclear localization is exaggerated in *hcf-1* loss of function mutants provides an important clue for the mechanism of HCF-1 inhibition of SKN-1. However, using the SKN-1::GFP reporter, we cannot discern whether the significant rise in nuclear SKN-1 is due to increased cytoplasm-nucleus shuttling, reduced nuclear export, elevated *skn-1* expression or protein stability. Given that HCF-1 is itself constitutively nuclear-localized and that it is a well-characterized transcriptional regulator, we suspect that HCF-1 may inhibit the expression of *skn-1* under stress. Nevertheless, we can not exclude the possibility that HCF-1 promotes the cytoplasmic export or degradation of SKN-1 protein to keep SKN-1 activity in check. It has been shown that SKN-1 is subject to post-translational ubiquitylation and subsequent proteasomal degradation mediated by core proteasome components (Kahn *et al.*, 2008; Choe *et al.*, 2009). Interestingly, we found from our microarray studies that the expression of many FBOX proteins involved in ubiquitin-mediated protein degradation is highly regulated by HCF-1 (Table 3.3). This implies that HCF-1 may promote proteasomal degradation of SKN-1. Future studies to elicit the exact mechanism by which HCF-1 regulates the levels of nuclear SKN-1 will be necessary.

In summary, we uncovered a novel functional interaction between HCF-1 and SKN-1, two major contributors to aging and organismal stress response processes. Our findings

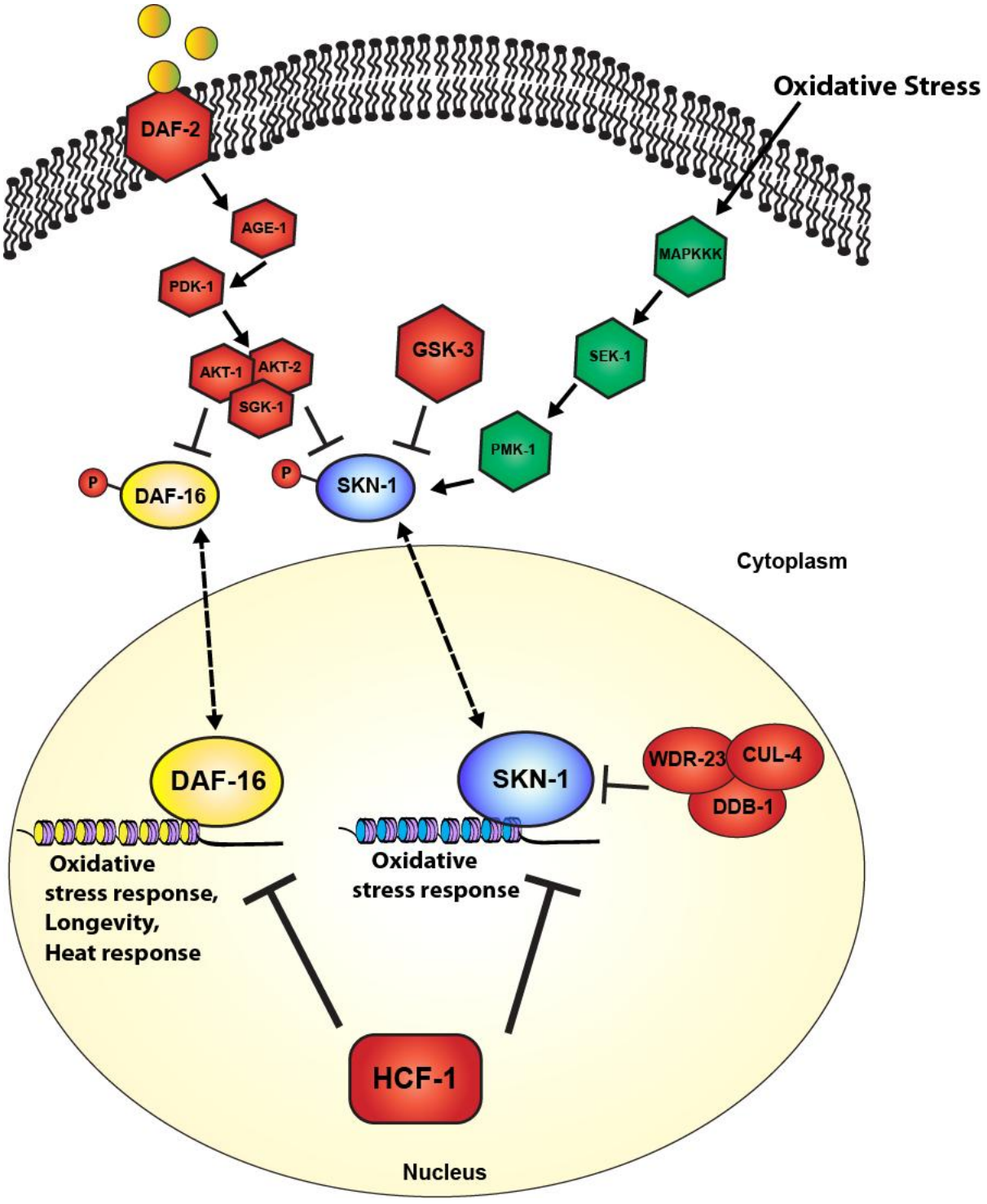


Figure 3.5. HCF-1 inhibits SKN-1 independently of DAF-16 to modulate defenses against oxidative stress.

further our knowledge of coordinated response mechanisms required to sense and fight against harmful toxic insults. The highly conserved nature of both HCF-1 and SKN-1 proteins raise the possibility that a similar regulatory relationship between mammalian HCF and Nrf factors may represent an important defense against oxidative stress-induced ailments such as cancer and cardiovascular disease.

MATERIALS AND METHODS

C. elegans strains

Strain stocks were maintained on Nematode Growth Medium (NGM) plates seeded with *E. Coli* OP50 bacteria at 16°C. Strains used were: N2 wild type, *hcf-1(pk924), daf-16(mgDf47),* IU162.1 *daf-16(mgDf47);hcf-1(pk924),* LD1002 *Ex[gcs-1::gfp pRF4 rol-6]* (An and Blackwell, 2003), CL2166 [*pAF15 gst-4::gfp::nls]* (Kahn *et al.*, 2008), *Ex[gst-7::gfp]* (Tullet *et al.*, 2008), LD1004 *Ex[skn-1b/c::gfp pRF4 rol-6]* (An and Blackwell, 2003); IU408.1 *hcf-1(pk924);skn-1b/c::gfp.*

RNAi

RNAi clones were grown overnight in the presence of tetracycline and carbeniccilin, cultures diluted tenfold the next day, grown to OD_{600} 0.8, induced with 4mM IPTG for 4 hours at 37°C, concentrated to a final concentration of OD_{600} 7.5, and seeded onto RNAi plates containing carbeniccilin and tetracycline. One day before use, RNAi bacteria on plates were re-induced by the addition of IPTG to 4mM. The RNAi construct targeting HCF-1 corresponds to the full-length *hcf-1* genomic sequence and is generated in our lab. *skn-1* RNAi spans the full-length *skn-1c* isoform and is a generous gift from Dr. Keith Blackwell, Harvard Medical School. *sgk-1* RNAi bacteria were obtained from the Ahringer RNAi library.

Stress and lifespan assays

For all experiments except Figure 3.1A, gravid adult parents were allowed to lay eggs overnight at 16°C and their synchronized progeny were switched to 25°C after hatching. Worms were subsequently transferred to plates containing FUDR at young adult stage to prevent reproduction.

For stress assays, paraquat and sodium arsenite (NaAs) (Sigma) were dissolved in water and *tert*-butyl hydroperoxide(*t*-BOOH) (Sigma) was diluted in decane before dispensing onto seeded plates to achieve a final concentration of 25mM or 50mM paraquat, 10mM NaAs, and 4mM *t*-BOOH. These chemicals were allowed to dry and diffuse overnight on plates before transferring animals. Stress plates contained FUDR throughout the rest of the experiment.

For Heat shock assay, worms were transferred into a 32°C incubator on day one of adulthood. The sides of the plates were covered by Parafilm to prevent excessive evaporation from the sides. Six small holes were drilled on the cap of the plates to allow for equilibration of humidity on the surface of the agar. The plates were placed in a single layer on the same shelf of the incubator to ensure minimal variability due to temperature fluctuations.

For lifespan assays, animals were additionally transferred to fresh RNAi plates (+FUDR) at Day 2, 4, and 8 of adulthood.

For all survival assays, worms were grown and assayed on triplicate plates per Strain+RNAi combination. All statistical analyses were performed using SPSS software Kaplan Meier survival and *p*-values were computed using log-rank statistics. Data from independent experiments were pooled whenever possible, such as when experiments were repeated under identical conditions, to improve statistical power.

RNA isolation, RT-qPCR, Microarrays

Total RNA was isolated from hypochlorite-synchronized young adult (YA) worms. Eggs were allowed to hatch in M9 buffer overnight at 16°C, and 600 L1 stage worms were plated

onto each of 5-6 10mm NGM plates seeded with 3-times concentrated OP50 bacteria. The synchronized populations were incubated at 25°C until they reached YA stage and harvested by washing off the plates with M9 buffer and freezing the worm pellet in liquid nitrogen. Total RNA was isolated using Tri-reagent (Molecular Research Center, Inc.) (Troemel *et al.*, 2006). For RT-qPCR, total RNA was reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad) to obtain cDNAs. SYBR Green quantitative PCR was performed on a Roche LightCycler 480 real time PCR machine to quantify cDNAs relative to a standard curve and normalized to *act-1*. *sod-3* and *act-1* primers have been described in (Li *et al.*, 2008). Other primers are: *skn-1* forward: 5'-GTAGCCGACGACGAAGAAGA-3', *skn-1* reverse: 5'-GGA TTGAGGTGTTGGACGAT-3', *gst-38* forward: 5'-AGCTTCCAATGCTCGAGGTA-3', *gst-38* reverse: 5'-GGCCAAGGAGTTGACTTGAG-3', *gst-12* forward: 5'GGAGTTCCGTTTGAGGATGA-3', *gst-12* revese: 5'-CGACGTTTAGGACAGGCATT-3', *gst-14* forward: 5'-GGAGTTCCGTTTGAGGATGA-3', *gst-14* reverse: 5'-AGCGATTTATGGCAGCAGAT-3', F56D5.3 forward: 5'-TGTGGAATTTGCTGAAACCA -3', F56D5.3 reverse: 5'-CCATTGCACCAGTTGTTCTG-3', K10H10.2 forward: 5'-CCGGAGAATAAGGGGAAACT-3', K10H10.2 reverse: 5'-GGCAATCTTGATGGAATCGT-3', *cyp-14A1* forward: 5'-CGGCAATTGTGTTGACTGAT -3', *cyp-14A1* reverse: 5'-TGATCGTGAACTGGCAGAAG-3', *gst-4* forward: 5'-CCGTTTTCTATGGAAGTGACG-3', *gst-4* reverse: 5'-CCCAAGTCAATGAGTCTCCAA-3'.

For microarrays, total RNA was purified with the RNeasy kit (Qiagen). cRNA synthesis/amplification, Cy3/Cy5 dye labeling, and hybridization onto Agilent 4X44K *C. elegans* oligonucleotide microarrays were performed as previously described (Shaw *et al.*, 2007).

Microarray analysis

Microarray slides were washed according to Agilent instructions after hybridization, and images were scanned using an Axon Instruments GenePix 4000B scanner (Axon Instruments, http://www.axon.com) (Pleiss *et al.*, 2007). The arrays were scanned at four different PMT settings to capture spots with low and high signal, and later combined to create a single dataset. Data were submitted to Princeton University MicroArray database (PUMA [http://puma.princeton.edu]) for subsequent processing. Normalization, filtering for array and spot quality, collapsing replicate spots to a mean value on PUMA generated $\log_2$ transformed fold change data for further analyses.

SAM analysis: Fold change data with no cutoff were submitted to Significance Analysis of Microarrays (SAM) (Tusher *et al.*, 2001). One class and two-class unpaired analysis were employed to identify genes similarly and divergently changed between *hcf-1(-)* and *skn-1(+)* datasets. A stringent criteria of False Discovery Rate (FDR) =0 was used. Significant changed genes were hierarchically clustered by Cluster3.0 (Eisen *et al.*, 1998)and displayed with Treeview (Eisen *et al.*, 1998).

Gene Ontology classification: Worm Base IDs (WBID) of each gene cluster generated by SAM and cluster were submitted to DAVID (http://david.abcc.ncifcrf.gov/) for Gene ontology enrichment analysis (Dennis *et al.*, 2003; Huang da *et al.*, 2009). Functional annotation clustering was performed with the default criteria.

GFP fluorescence scoring

Induction of GFP fluorescence in GCS-1::GFP, GST-4::GFP, GST-7::GFP or SKN-1::GFP strains was measured as described in (An and Blackwell, 2003; Tullet *et al.*, 2008). For SKN-1::GFP, "Low" indicates that fluorescence is only observed at the two ASI neurons, "Medium" signal depicts expression in anterior or posterior, or both intestinal nuclei, and "High" indicates visible fluorescence throughout the entire length of the intestine. For SKN-1 target gene reporters, the scoring system was identical to that of SKN-1::GFP except "Low"

represents GFP signal only detectable in the head/pharynx of the animal. In all cases, synchronized L4 stage worms were visualized. The experiments were conducted blindly where the experimenter did not know which RNAi or stress condition they were scoring.

ACKNOWLEDGEMENTS

We thank the *Caenorhabditis elegans* Genetics Center (CGC) for strains; Keith Blackwell (Harvard Medical School) for providing GFP strains and *skn-1* RNAi construct; Jeffrey A. Pleiss (Cornell University) for the use of GenePix scanner and help with microarray data acquisition; Crystal Shana Faith Conn (Cornell University) for help with scoring *gst-4::gfp* and *gst-7::gfp* experiments; members of the Lee lab and Cornell worm group for helpful suggestions.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Aging is a complex biological process which is governed by combinatorial effects of environmental and genetic factors. Due to the initial discovery of conserved longevity pathways such as the insulin/IGF-1-like signaling cascade (Kenyon *et al.*, 1993; Gottlieb and Ruvkun, 1994), aging research has picked up speed at an exponential pace within the last several decades. An extensive effort has so far uncovered numerous lifespan modulatory pathways, factors, and interventions. It is of immense interest to fully comprehend the interactions and mechanisms that influence aging and age-associated diseases which can ultimately translate into therapies and interventions to both prolong life and prevent the development of age-related disorders. My work has focused on studying the functions of a relatively newly identified longevity factor, HCF-1, that represents one branch of the complex regulatory network contributing to aging.

I have described and characterized two novel HCF-1 interactions with highly conserved longevity and stress response determinants SIR-2.1, mammalian SIRT1 homolog, and SKN-1, mammalian Nrf ortholog in *C. elegans.* My work led to the discovery of SIR-2.1 as an upstream inhibitor of HCF-1, where this regulatory interaction converges on DAF-16 to alter its transcriptional activity essential for longevity and oxidative stress response processes (Chapter 2). An analogous link between mammalian SIRT1 and HCF proteins is uncovered by Terri Iwata (*Rizki et al*, 2011, *PLoS Genetics*, accepted, and unpublished results), highlighting the conserved nature of HCF-1 functions in longevity and stress responses. I further demonstrated that HCF-1 represses transcactivation by SKN-1 in parallel to DAF-16 specifically under oxidative stress conditions (Chapter 3). Even though my findings shed light onto the mechanisms by which HCF-1 modulates lifespan and stress responses, my conclusions inevitably evoke many additional questions that need to be addressed in the

future.

We began investigating the interactions between HCF-1 and DAF-16 cofactors based on the hypothesis that *C. elegans* HCF-1, as is the case for mammalian HCF-1, engages with various transcriptional complexes to exert its effects on worm lifespan and stress responses. Besides SIR-2.1, I extended my studies to examine whether additional DAF-16 cofactors are involved in the control of DAF-16 activity by HCF-1. As discussed in Appendix I, I illustrated that the mammalian SMEK1 homolog, SMK-1, but not the β-catenin homolog BAR-1, works downstream of HCF-1 to promote DAF-16-mediated transcription of genes important for prolonging lifespan and enhancing oxidative stress resistance. It will be important to follow up my observations with additional molecular and global gene expression analyses to understand the molecular mechanism whereby HCF-1 inhibits an SMK-1/DAF16 complex and to unveil the transcriptional outcome of SMK-1/DAF-16 response to HCF-1. Furthermore, multiple additional DAF-16 coregulators have been identified within the last few years (see Chapter 1 for details). Currently, the functional and molecular interactions between these numerous nuclear DAF-16 cofactors are utterly elusive. To gain insight into the undoubtedly complex interaction network around DAF-16, systematic functional and molecular assays are necessary. In fact, studies are currently under way in our lab to dissect out the interaction network surrounding DAF-16 and HCF-1.

Despite a substantial body of evidence suggesting that HCF-1 engages downstream regulators to affect lifespan and stress response in worms, the upstream signals that control HCF-1 functions have not been explored. It is well established that HCF-1 proteins are subject to post-translational modifications such as phosphorylation and acetylation (Cai *et al.*, 2010; Wysocka *et al.*, 2001; Wang *et al.*, 2007). In a mass spectrometry analysis of HCF-1 bound proteins, several interesting *C. elegans* HCF-1 interactors including *ulp-2,* a protease, and *sin-3* and *hda-1,* deacetylases, have been identified (unpublished results by Christian G. Riedel). In the future, a targeted screen designed to identify protein modifiers with direct

impact on HCF-1's phosphorylation, acetylation, and other modifications may aid in unearthing upstream regulators of HCF-1 function. Determining the upstream components modulating HCF-1's roles with respect to lifespan and stress response processes will contribute to understanding the molecular determinants of HCF-1 activity.

Another interesting question that emerged from my findings linking HCF-1 and SKN-1 in oxidative stress response is whether mammalian HCF proteins are involved in responding to oxidative challenges through inhibiting Nrf factors. Given the high degree of conservation between mammalian and *C. elegans* HCF proteins, the functional interaction between HCF-1 and SKN-1 likely exists in mammals. One path towards answering the involvement of HCFs in retaliation against oxidative stress is to test the effects of altered HCF expression on the survival of mammalian cells upon exposure to oxidants. To test whether HCFs repress Nrf activation, the effects of altered HCF-1 or HCF-2 levels on the nuclear localization as well as the transcriptional activity of Nrf factors in mammalian cells could be measured. Understanding the roles of HCF factors in mechanisms that cope with oxidative stress in mammalian cells and tissues will undeniably implicate HCFs as therapeutic targets for oxidative-stress-associated diseases such as cancer, cardiovascular disease, and neurodegenerative disorders .

APPENDIX I

HCF-1 REQUIRES THE DAF-16 COFACTOR SMK-1 BUT NOT BAR-1 TO REGULATE DAF-16 ACTIVITY[3]

As previously described, HCF-1 is known to engage in large protein networks to accomplish transcriptional repressor or activator functions (Wysocka and Herr, 2003; Wysocka *et al.*, 2003). To broaden our understanding of the molecular interactors of HCF-1 protein necessary for it to execute its longevity and stress response functions, we expanded our genetic interaction studies to known DAF-16 cofactors. As detailed in Chapter 1.2.2.2 and 1.2.2.3, SMK-1 and BAR-1 represent two of the first few DAF-16 coactivators to have been discovered. While both SMK-1 and BAR-1 are necessary for DAF-16-mediated longevity and oxidative stress response, SMK-1 additionally modulates DAF-16 activation during UV and pathogen stress in the context of reduced IIS (Essers *et al.*, 2005; Wolff *et al.*, 2006).

In order to determine whether HCF-1 functionally interacts with SMK-1 and BAR-1 to influence DAF-16 activity, we conducted genetic epistasis analyses in lifespan, oxidative stress, and transcription. We show that *smk-1* is necessary for the long-lifespan, enhanced survival during oxidative stress, and elevated expression of a well-characterized direct target of DAF-16, superoxide dismutase-3 (*sod-3).* On the other hand, *bar-1* inactivation by RNAi knockdown does not suppress the prolonged lifespan, oxidative stress resistance, and induced *sod-3* expression exhibited by *hcf-1* mutants. Collectively, our data are consistent with a hypothesis that SMK-1, but not BAR-1, works downstream of HCF-1 to govern DAF-16 activity.

[3] The *smk-1* RNAi treatment of *hcf-1(pk924)* mutants in lifespan assays has previously been performed by Ji Li and Terri Iwata with similar results.

RESULTS

smk-1, but not *bar-1,* contributes to the longevity and enhanced oxidative stress survival of *hcf-1* mutants

To examine the effects of inactivating *smk-1* and *bar-1* on *hcf-1*-mutant associated longevity and stress response phenotypes, we performed double stranded RNA-mediated knockdown of *smk-1* and *bar-1* in *hcf-1(pk924)*- harboring worms, and assayed their lifespan and survival after exposure to an oxidative-stress inducing agent, paraquat. We observed that *smk-1* knockdown substantially augmented *hcf-1* mutant longevity and oxidative stress resistance, indicating that *smk-1* works downstream of HCF-1 (Figures AI.1A,C; Tables AI1.A,C). In contrast, *bar-1* inactivation did not affect *hcf-1(pk924)*-associated lifespan and oxidative stress resistance any more than it affected wild-type (Figure AI.1B,D; Table AI.1B,D). We were unable to employ *smk-1* and *bar-1* mutant strains for our epistasis experiments since the only available *smk-1* mutant strain displays a high degree of embryonic lethality and *bar-1(ga80)* strain causes developmental defects (Eisenmann *et al.*, 1998). Our findings reveal that while the DAF-16 coactivator SMK-1 mediates longevity and oxidative stress response functions of HCF-1, BAR-1 does not act downstream of HCF-1 to execute these functions.

smk-1 is required for the *daf-16*-mediated transcriptional response to *hcf-1* deficiency

We have previously shown that a subset of DAF-16 target genes are induced or repressed in the absence of *hcf-1* (Li *et al.*, 2008). Both SMK-1 and BAR-1 have been reported to promote transactivation of DAF-16's downstream targets (Essers *et al.*, 2005; Wolff *et al.*, 2006). To determine whether SMK-1 or BAR-1 participate in the regulation of DAF-16's transcriptional activity by HCF-1, we assayed the expression of a well-characterized DAF-16 target gene, *sod-3.* We exploited a frequently used *sod-3* transcriptional reporter strain, where the worms possess a transgene expressing GFP fused to

the *sod-3* promoter (*psod-3::gfp).* *hcf-1* mutation in the p*sod-3::gfp* strain results in visibly elevated levels of GFP in the vulva, multiple neurons, intestinal, and hypodermal cells of the animal (Figure AI.2A). We found that depleting *daf-16* and *smk-1* attenuated the enhanced *sod-3* expression in *hcf-1* mutants. On the contrary, *bar-1* knockdown did not reduce *sod-3* levels (Figure AI.2A). We next assessed the effects of *smk-1* RNAi on the levels of endogenous *sod-3* and C32H11.4, a gene whose transcription is repressed by DAF-16 in the absence of *hcf-1* (Li *et al.*, 2008). RT-qPCR analysis showed that *smk-1* RNAi suppressed the increased *sod-3* and decreased C32H11.4 mRNA levels in *hcf-1*-impaired worms (Figure AI.2B). Our transcriptional data illustrate that SMK-1 is necessary to facilitate DAF-16's ability to regulate its downstream target genes in response to the disruption of HCF-1 function.

In conclusion, our results implicate SMK-1 as an integral partner of DAF-16 downstream of HCF-1 regulation. Conversely, our findings suggest that BAR-1 likely acts independently of HCF-1 to modulate DAF-16's activation. Future studies to further investigate the genetic and molecular interactions between HCF-1 and SMK-1 will shed light onto the mechanism by which HCF-1 exerts its effects on longevity and stress response through DAF-16.

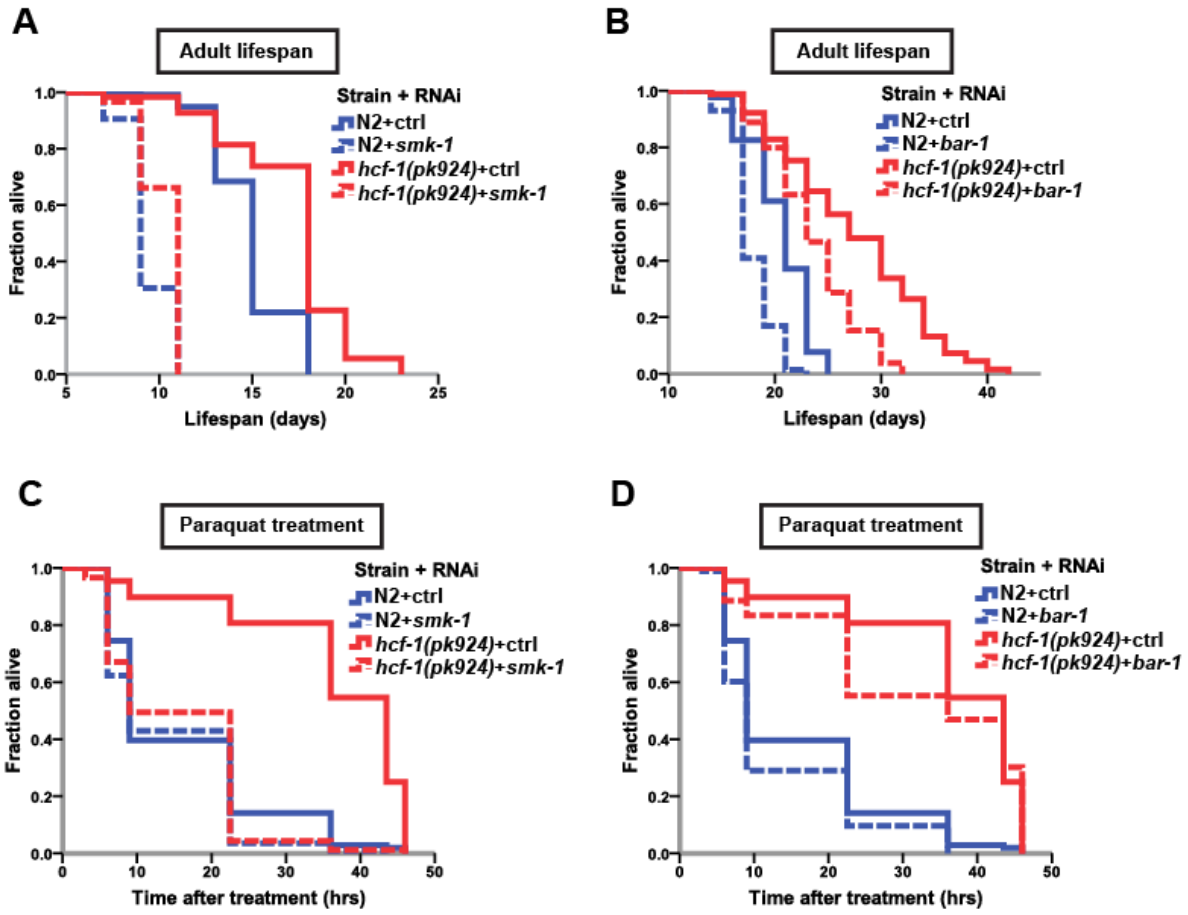


Figure AI.1. *smk-1* contributes to the extended lifespan and heightened oxidative stress resistance of *hcf-1* mutants whereas *bar-1* does not. (A) RNAi targeting a non-specific sequence (control, L4440) or *smk-1* was initiated at embryo stage and the worms of indicated genotypes were grown at 25°C. Survival of animals was recorded every other day starting from adulthood. This experiment was done once by myself but similar results were obtained by multiple other people. (B) *bar-1* RNAi was started at L4 stage to avoid adverse developmental effects. The experiment was carried out at 20°C. Only one experiment was performed. (C) Worms subjected to *smk-1* or control RNAi were grown until Day two of adulthood at 25°C and subsequently incubated in M9 buffer containing 200mM paraquat. Survival graph is generated by combining data from two independent repeats. (D) Experiment was carried out as in (C). Data pooled from two independent experiments are displayed.

Table AI.1A

Epistasis between *hcf-1(pk924)* and *smk-1* RNAi in lifespan

Strain + RNAi	Mean Lifespan + SEM(Days)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. *hcf-1(pk924)* + ctrl	% effect on N2 + ctrl	% effect by *hcf-1(pk924)* vs. corresponding N2+RNAi
N2 + ctrl	14.9 ± 0.2	115		<0.001		
N2 + *smk-1*	9.4 ± 0.1	106	<0.001	<0.001	-37	
hcf-1(pk924) + ctrl	17.3 ± 0.4	60	<0.001		16	16
hcf-1(pk924) + *smk-1*	10.3 ± 0.1	90	<0.001	<0.001	-31	9

All survival analyses were done using SPSS software, Kaplan Meier analysis and log-rank test to compute *p*-values. *p*-value <0.05 is considered statistically significant.

Table AI.1B

Epistasis between *hcf-1(pk924)* and *bar-1* RNAi in lifespan

Strain + RNAi	Mean Lifespan + SEM(Days)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. *hcf-1(pk924)* + ctrl	% effect on N2 + ctrl	% effect by *hcf-1(pk924)* vs. corresponding N2+RNAi
N2 + ctrl	20.5 ± 0.3	89		<0.001		
N2 + *bar-1*	18.0 ± 0.2	95	<0.001	<0.001	-13	
hcf-1(pk924) + ctrl	27.6 ± 0.8	93	<0.001		34	34
hcf-1(pk924) + *bar-1*	23.7 ± 0.5	92	<0.001	<0.001	15	32

Experiment was carried out at 20°C.

Table AI.1C

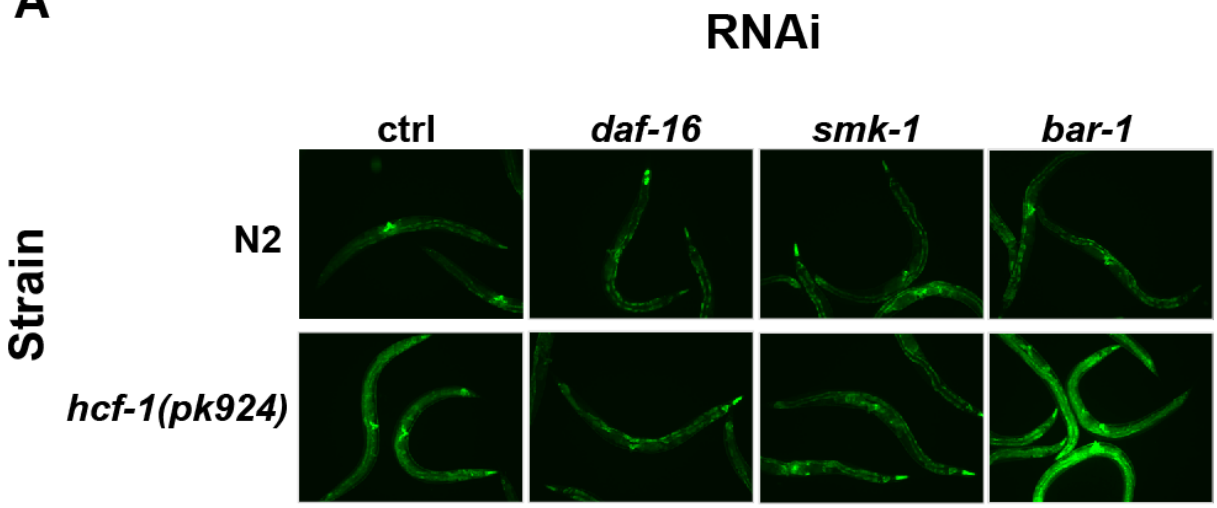
Epistasis between *hcf-1(pk924)* and *smk-1* or *bar-1* RNAi in paraquat

Strain + RNAi	Mean Survival + SEM(Hrs)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. *hcf-1(pk924)* + ctrl	% effect on N2 + ctrl	% effect by *hcf-1(pk924)* vs. corresponding N2+RNAi
N2 + ctrl	15.8 ± 1.1	106		<0.001		
N2 + *smk-1*	14.2 ± 0.8	114	0.145	<0.001	-10	
N2 + *bar-1*	13.0 ± 1.0	93	0.033	<0.001	-18	
hcf-1(pk924) + ctrl	36.6 ± 1.3	88	<0.001		132	132
hcf-1(pk924) + *smk-1*	15.3 ± 1.0	91	0.611	<0.001	-3	8
hcf-1(pk924) + *bar-1*	31.6 ± 1.5	96	<0.001	0.547	100	143

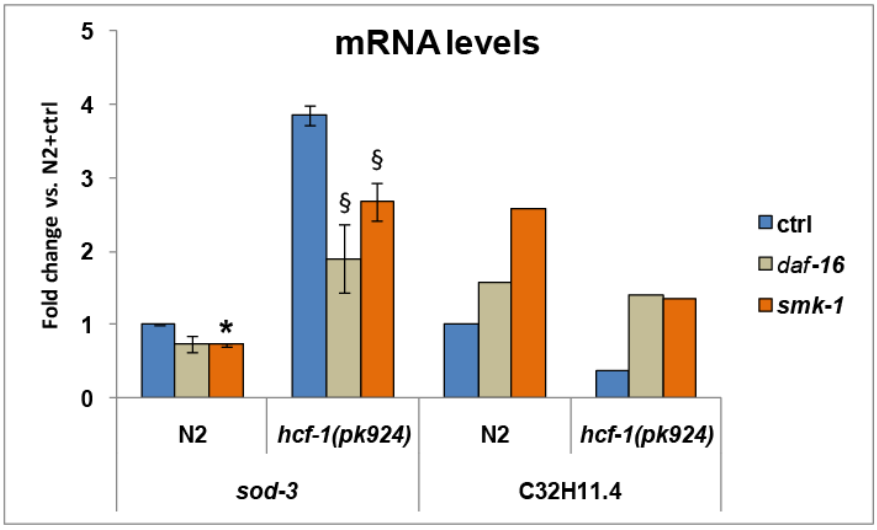
Data pooled from two independent experiment are shown. All survival analyses were done using SPSS software, Kaplan Meier analysis and log-rank test to compute *p*-values. *p*-value <0.05 is considered statistically significant.

Figure AI.2. Inactivating *smk-1* suppresses the altered expression of two *daf-16* target genes, *sod-3* and C32H11.4 in *hcf-1* mutants. (A) Animals expressing a transcriptional *sod-3::gfp* reporter were grown on dsRNA-expressing bacteria at 25°C for two generations. The GFP fluorescence of young adult worms was visualized and representative images from each strain+RNAi combination are displayed. (B) Total RNA was purified from worms treated with ctrl, *daf-16,* and *smk-1* RNAi and mRNA levels quantified by RT-qPCR. Measurements were normalized to total actin levels. For *sod-3,* data are averaged from two independent experiments and displayed as +/- SEM. C32H11.4 levels reflect data from a single experiment. *denotes *p*-value<0.05 compared to N2+ctrl, §denotes *p*-value<0.05 compared to *hcf-1(pk924)*+ctrl.

A


RNAi
ctrl
daf-16
smk-1
bar-1
Strain
N2
hcf-1(pk924)

B


mRNA levels
Fold change vs. N2+ctrl
5
4
3
2
1
0
*
§
§
ctrl
daf-16
smk-1
N2
hcf-1(pk924)
N2
hcf-1(pk924)
sod-3
C32H11.4

APPENDIX II

IDENTIFYING POTENTIAL *C. ELEGANS* HCF-1 INTERACTING FACTORS THROUGH A TARGETED RNAi SCREEN

Mammalian HCF-1 participates in numerous protein complexes to regulate transcription. In a mass spectrometry analysis of HCF-1 interacting proteins, multiple chromatin modifying complex components were discovered (Wysocka *et al.*, 2003). Namely, Sin3 histone deacetylase and Set1/Ash2 and MLL histone methyltransferase complexes are major interactors of HCF-1 (Wysocka *et al.*, 2003; Yokoyama *et al.*, 2004; Tyagi *et al.*, 2007). Since the physiological functions of HCF-1 are just beginning to emerge in *C. elegans*, we hypothesized that the worm HCF-1 homolog may associate with conserved chromatin modifiers to impact gene expression. To test this, we obtained all the available RNAi constructs corresponding to the *C. elegans* homologs of mammalian HCF-1 binding partners (Ahringer and Vidal RNAi libraries) (Table II.1), knocked down each factor and monitored their effects on the transcription of superoxide dismutase-3 (*sod-3),* a well-characterized HCF-1/DAF-16 target gene (Li *et al.*, 2008). We reasoned that if any of the chromatin modifying factors is working with HCF-1 to influence the expression of *sod-3,* we should observe a change in *sod-3* expression levels upon attenuating the function of that factor. Indeed, inactivating the histone deacetylase *sin-3,* which is classically associated with transcriptional repression, as well as F21H12.1, the homolog of RbBP5 WD40 repeat protein that is part of the MLL histone methyltransferase complex, resulted in enhanced *sod-3* expression comparable to that induced by *hcf-1* knockdown. We also observed that *sin-3* and F21H12.1 further magnified the *sod-3* -induction of *daf-2(e1370)* mutants, another trend displayed by *hcf-1* knockdown. Interestingly, depleting the MLL components *set-1* and *set-16* produced a specific *sod-3* elevation in the absence of *daf-2,* suggesting a possible crosstalk with the IIS.

Overall, our RNAi screen revealed that *sin-3* histone deacetylase and MLL histone methyltransferase complexes likely play roles in the expression of the *sod-3* gene. It will be of interest to test the functions of these components in longevity and stress response determination as well as perform epistasis and molecular analyses to explore possible interactions with HCF-1.

Table AII.1			First generation RNAi			2nd generation RNAi	
HCF-1 interacting Complex	Components	*C. elegans*	EXP1 (*sod-3::gfp*)	EXP2 (*sod-3::gfp*)	EXP2 (*daf-2(e1370)* ; *sod-3::gfp*)	EXP3 (*sod-3::gfp*)	EXP3 (*daf-2(e1370)* ; *sod-3::gfp*)
Sin3A	Sin3A	*sin-3 (pqn-28)*	↑ Whole body			↑ Vulva & whole	↑ **Whole body**
	Sds3						
Set1/Ash2	Set1	*set-2*	↑ Maybe vulva	No effect	↑ slight increase	No effect	↑ slight increase
	Ash2	Y17G7B.2	N/A				
	WDR5	*tag-125*	No effect	No effect	No effect	No effect	No effect
MLL complex	Set1	*set-2*	Maybe vulva	No effect	↑ slight increase	No effect	↑ slight increase
	Ash2	Y17G7B.2					
	WDR5	*tag-125*	No effect	No effect	No effect	No effect	No effect
	RbBP5(WD40)	F21H12.1	↑ Whole body	↑ Maybe vulva &tail?	↑ Whole body	↑ Maybe whole body	↑ Whole body
	MLL1 or 4	*set-2 ?*					
	MLL 2 or 3	*set-16*	No effect	No effect	↑ Whole body	No effect	↑ Whole body
COMPASS	Set1	*set-2*	↑ Maybe vulva	No effect	↑ slight increase	No effect	↑ slight increase
	Others	WDR5 (tag-125)	No effect	No effect	No effect	No effect	No effect
		dpy-30		No effect	No effect	No effect	No effect
		mes-4		No effect	No effect	↑ **Vulva and** body	↑ **Vulva and** body
Controls		*daf-2*	↑ Whole body	↑ Whole body	↑ **Whole body**	↑ Whole body	↑ **Whole body**
		hcf-1		↑ Vulva & tail	↑ **Whole body**	↑ Vulva & tail	↑ **Whole body**
		daf-16	↓ Vulva	↓ Vulva	↓ very very dim	↓ Vulva	↓ very very dim

Worms carrying a transcriptional *sod-3::gfp* reporter or *daf-2(e1370);sod-3::gfp* were fed RNAi bacteria to knockdown the *C. elegans* homologs of indicated mammalian HCF-1-interacting partners. N/A=The RNAi construct was not available. The increase or decrease in GFP fluorescence was determined based on the majority of a population of worms (N>50). Green arrows represent increases and black arrows decreases in GFP signal compared to the L4440 treated controls and the region of the body where the change occurred is also listed. The thickness of the arrow and size of the text reflects the degree of GFP fluorescense (the bigger the arrow/text, the higher the signal) relative to control. EXP=Experiment.

APPENDIX III

EXAMINING THE FUNCTIONS OF *C. ELEGANS* POLY(ADP-RIBOSE) POLYMERASES IN LONGEVITY

Poly(ADP-ribose) metabolism pathway has been implicated in aging in different species. Poly(ADP-ribose) (PAR) is a post-translational modification of proteins. PARylation is involved in the regulation of a broad range of biological processes including DNA damage response, cell death, chromatin structure, telomere maintenance, gene expression, and metabolism (Krishnakumar and Kraus, 2011; Luo and Kraus, 2011; Kim *et al.*, 2005). A dynamically regulated balance of PAR levels is critical for the proper functioning of cells, particularly under DNA damage-inducing conditions. Two classes of enzymes achieve the proper balance of PAR levels: Poly(ADP-ribose) polymerase (PARP), which catalyzes the polymerization of PAR chains using donor NAD+ molecules, and Poly(ADP-ribose) glycohydrolase (PARG), which degrades the PAR chains. PAR metabolism has been implied to have roles in longevity (Burkle *et al.*, 2005). Due to their critical functions in genome maintenance, PARPs could have beneficial effects on the long-term survival of an organism. One mammalian study suggested a positive correlation between high PARP activity and long lifespan (Grube and Burkle, 1992). Partly because of their co-dependence on NAD(+) metabolism, PARP enzymes have been shown to be functionally linked to the important longevity determinant, Sir2 (Pillai *et al.*, 2005). Two recent studies uncovered that deletion of two mammalin PARP family members, PARP-1 and PARP-2, exhibit elevated mitochondrial energy expenditure and protection from diet-induced obesity in mice (Bai *et al.*, 2011; Bai *et al.*, 2011). Interestingly, both PARPs accomplish their effects on mouse metabolism through inhibiting the activation and expression of mammalian SIRT1, suggesting that PARP proteins may indeed contribute to longevity through interactions with SIRT1 (Bai *et al*, 2011.; Bai *et al.*, 2011). However, the evidence

suggesting a role for PAR metabolism in aging has been only correlative and a possible direct effect of this pathway on aging has not been systematically addressed.

Poly(ADP-ribosyl)ation is a very conserved process and similar to many other organisms, PARP and PARG homologs exist in *C. elegans*. There are four PARP and two PARG homologs in the worm. Therefore, by taking advantage of the powerful genetic tools in *C. elegans,* I investigated a possible role for PAR metabolism in modulating *C. elegans* lifespan. My project focused on studying the PARP enzymes in relation to aging. My goal had been to understand how altered PARP expression and/or activity affected lifespan and the mechanism behind these possible effects. After rigorous experimentation, my data suggest that inactivating or overexpressing PARP homologs in *C. elegans* does not result in consistent and robust effects on longevity. A parallel study carried out by Dr. Zoey Ni in our lab, examining the effects of PARG enzymes on aging, similarly deduced a lack of major involvement of PAR metabolism in longevity determination.

RESULTS

Inactivating the PARP genes one at a time or in combination does not result in robust changes in the longevity of *C. elegans*

In order to explore the effects of reducing PARP function in the rate of aging in worms, we diminished PARP levels and measured the survival of adult animals. There are four PARP homologs in *C. elegans*: *pme-1*(huPARP-1), *pme-2* (huPARP-2), *pme-5* (huTankyrase-1), and *pme-6* (pseudogene), which arose from a duplication of *pme-1*. We obtained *C. elegans* mutants harboring a deletion in either the *pme-1* or *pme-5* genes and backcrossed these strains several times to eliminate possible background mutations. Our lab also obtained or produced RNAi constructs targeting each of the PARP genes. We performed lifespan assays in which we either inactivated each PARP gene alone by mutation or RNAi, or to get around possible redundancy between PARPs, we knocked down PARP proteins in the

mutant strains (Tables AIII.1A-H). To ensure that we captured the possible influences of growing worms at different temperatures on *pme* inactivation, we also performed our lifespan experiments at 20°C and 25°C (Tables AIII.1A-H). In addition, we conducted several RNAi experiments using an RNAi-sensitive mutant background, *rrf-3(pk1426)* (Simmer *et al.*, 2002) (Table AIII.1E). We further compared assaying lifespan on live versus killed bacteria, a condition which is known to influence the longevity phenotypes of several mutants (Mallo *et al.*, 2002) (data not shown). Collectively, attenuating PARP levels in worms did not yield any consistent and strong lifespan phenotypes.

Overexpressing *pme-1* or *pme-5* in *C. elegans*

In parallel to the *pme* inactivation experiments, we sought to determine whether activating PARPs by overexpression of *pme-1* or *pme-5* caused longevity effects. We generated lines harboring low or high numbers of *pme-1* gene copies through microparticle bombardment and microinjection, respectively. We additionally created a low-copy overexpressor strain of *pme-5*. Using quantitative PCR, we measured the genomic copy number of *pme-1* overexpressor strains, and chose strains with the largest number of integrated *pme-1* copies for our subsequent lifespan assays (Figure AIII.1A). We next performed survival assays measuring the lifespan of PARP overexpressing strains as compared to their corresponding transgenic controls. Consistent with our findings that depleting PARPs does not yield major alterations in the lifespan of *C. elegans*, we failed to observe any noticeable changes in the lifespans of PARP overexpressing worms (Tables AIII.2A-C). However, our lifespan results are shadowed by the finding that *pme-1* mRNA levels were reduced despite the presence of multiple genomic copies of *pme-1* gene (Figure AIII.1B). We speculate that this transgenic silencing of the *pme-1* gene may have occurred as a result of self-regulation of PARP expression levels.

Table AIII.1A

***pme* mutant lifespan at 20°C**

Strain	Mean Lifespan + SEM(Days)	Total N	*p*-value vs. N2	% effect on N2
N2	18.4 ± 0.3	249		
pme-1(ok988)	18.6 ± 0.3	202	0.814	1
pme-5(ok446)	16.1 ± 0.2	177	<0.001	-13

Data are pooled from four independent experiments, representing three independent repeats for *pme-1(ok988)* and three repeats for *pme-5(ok446).* Experiments were carried out at 20°C. All survival analyses were done using SPSS software, Kaplan Meier analysis and log-rank test to compute *p*-values. *p*-value <0.05 is considered statistically significant.

Table AIII.1B

***pme* mutant lifespan at 25°C**

Strain	Mean Lifespan + SEM(Days)	Total N	*p*-value vs. N2	% effect on N2
N2	14.8 ± 0.2	286		
pme-1(ok988)	13.5 ± 0.2	146	<0.001	-9
pme-5(ok446)	14.3 ± 0.2	210	0.033	-3

Data from three independent experiments with two repeats for *pme-1* mutant and three repeats for *pme-5* mutant are combined. Experiments were carried out at 25°C.

Table AIII.1C

***pme* RNAi lifespan at 20°C - N2 background**

Strain + RNAi	Mean Lifespan + SEM(Days)	Total N	*p*-value vs. N2 + ctrl	% effect on N2 + ctrl
N2 + ctrl	20.8 ± 0.2	298		
N2 + *pme-1*	21.3 ± 0.2	174	0.042	2
N2 + *pme-2*	22.3 ± 0.2	345	<0.001	7
N2 + *pme-1/2*	22.6 ± 0.2	186	<0.001	9
N2 + *pme-5*	22.6 ± 0.1	183	<0.001	8

Data from three independent experiments representing three repeats for *pme-2* RNAi and two repeats for the rest are combined. *pme-1/2* indicates that the worms were exposed to RNAi prepared by mixing *pme-1* and *pme-2* dsRNA-expressing bacteria 1:1. Experiments were carried out at 20°C.

Table AIII.1D

***pme* RNAi lifespan at 25°C - N2 background**

Strain + RNAi	Mean Lifespan + SEM(Days)	Total N	*p*-value vs. N2 + ctrl	% effect on N2 + ctrl
N2 + ctrl	13.6 ± 0.2	66		
N2 + *pme-1*	14.6 ± 0.2	121	0.003	7
N2 + *pme-2*	15.4 ± 0.3	108	<0.001	13
N2 + *pme-5*	16.0 ± 0.2	160	<0.001	18

Data from one experiment is displayed. Experiment was done at 25°C.

Table AIII.1E

***pme* RNAi lifespan at 20°C - *rrf-3* background**

Strain + RNAi	Mean Lifespan + SEM(Days)	Total N	*p*-value vs. N2 + ctrl	% effect on N2 + ctrl
rrf-3(pk1426) + ctrl	18.0. ± 0.2	173		
rrf-3(pk1426) + *pme-1*	18.8 ± 0.2	65	0.199	-1
rrf-3(pk1426) + *pme-2*	18.7 ± 0.2	255	0.002	4
rrf-3(pk1426) + *pme-1/2*	17.6 ± 0.3	78	0.021	4
rrf-3(pk1426) + *pme-5*	18.3 ± 0.3	88	0.178	-2

Data from two experiments for *pme-2* RNAi and one experiment for the rest are displayed. Experiment was done at 20°C.

Table AIII.1F

***pme-1(ok988)+ pme* RNAi lifespan at 20°C**

Strain + RNAi	Mean Lifespan + SEM(Days)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. *pme-1(-)* + ctrl	% effect on N2 + ctrl	% effect on *pme-1(-)* + ctrl
N2 + ctrl	20.9 ± 0.3	91		0.847		0
N2 + *pme-1*	21.0 ± 0.3	94	0.936	0.793	0	0
N2 + *pme-2*	24.5 ± 0.4	93	<0.001	<0.001	17	17
N2 + *pme-1/2*	22.9 ± 0.3	95	<0.001	<0.001	10	9
N2 + *pme-5*	22.3 ± 0.3	97	<0.001	0.002	7	6
pme-1(ok988) + ctrl	21.0 ± 0.2	92	0.847		1	
pme-1(ok988) + *pme-1*	20.5 ± 0.2	88	0.165	0.108	-2	-2
pme-1(ok988) + *pme-2*	22.0 ± 0.3	117	0.011	0.007	5	5
pme-1(ok988) + *pme-1/2*	21.7 ± 0.2	92	0.141	0.104	4	3
pme-1(ok988) + *pme-5*	21.1 ± 0.1	94	0.678	0.602	1	1

Data from one experiment is shown. Experiment was carried out at 20°C.

Table AIII.1G

***pme-5(ok446)+ pme* RNAi lifespan at 20°C**

Strain + RNAi	Mean Lifespan + SEM(Days)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. *pme-5(-)* + ctrl	% effect on N2 + ctrl	% effect on *pme-5(-)* + ctrl
N2 + ctrl	15.8 ± 0.2	125		<0.001		11
N2 + *pme-1*	14.6 ± 0.2	121	<0.001	0.756	-8	2
N2 + *pme-2*	15.4 ± 0.3	108	0.277	0.007	-3	7
N2 + *pme-5*	16.0 ± 0.2	160	0.700	<0.001	1	12
pme-5(ok446) + ctrl	14.3 ± 0.3	113	<0.001		-10	
pme-5(ok446) + *pme-1*	14.7 ± 0.2	159	<0.001	0.401	-7	3
pme-5(ok446) + *pme-2*	14.8 ± 0.2	134	0.002	0.346	-7	3
pme-5(ok446) + *pme-5*	15.2 ± 0.3	79	0.100	0.049	-4	6

Data from one experiment is shown. Experiment was carried out at 25°C.

Table AIII.1H

***pme-1(ok988);pme-5(ok446)* + *pme* RNAi lifespan at 20°C**

Strain + RNAi	Mean Lifespan + SEM(Days)	Total N	*p*-value vs. N2 + ctrl	*p*-value vs. *pme-1(-); pme-5(-)* + ctrl	% effect on N2 + ctrl	% effect on *pme-1(-); pme-5(-)* + ctrl
N2 + ctrl	21.4 ± 0.2	112		0.689		0
N2 + *pme-1*	21.7 ± 0.4	80	0.112	0.382	1	1
N2 + *pme-2*	22.5 ± 0.3	65	0.003	0.030	5	5
N2 + *pme-1/2*	22.3 ± 0.3	91	0.004	0.038	4	4
N2 + *pme-5*	22.8 ± 0.3	86	<0.001	0.002	7	6
pme-1(-);pme-5(-) + ctrl	21.4 ± 0.3	80	0.689		1	
pme-1(-);pme-5(-) + *pme-1*	22.1 ± 0.4	74	0.464	0.800	2	1
pme-1(-);pme-5(-) + *pme-2*	22.2 ± 0.3	65	0.042	0.179	3	3
pme-1(-);pme-5(-) + *pme-1/2*	21.2 ± 0.4	73	0.034	0.155	4	3
pme-1(-);pme-5(-) + *pme-5*	21.1 ± 0.1	71	0.986	0.782	-1	-1

Data from one experiment is shown. Experiment was carried out at 20°C.

Table AIII.2A

***pme* low-copy overexpression lifespan at 20°C**

Strain	Mean Lifespan + SEM(Days)	Total N	*p*-value vs. N2	% effect on N2
ctrl	16.4 ± 0.2	752		
pme-1(O/E)	16.9 ± 0.2	1224	0.073	3
pme-5(O/E)	14.2 ± 0.4	169	<0.001	-15

Data are pooled from three independent experiments, representing three independent repeats for *pme-1(O/E)* and one experiment for *pme-5(O/E).* In addition, data from independent overexpression or control lines are combined. Control transgenic strain is constructed by bombarding the *unc-119*-containing contstruct used to build *pme-1* and *pme-5* plasmids. Experiments were carried out at 20°C. All survival analyses were done using SPSS software, Kaplan Meier analysis and log-rank test to compute *p*-values. *p*-value <0.05 is considered statistically significant.

Table AIII.2B

***pme-1* low-copy overexpression lifespan at 25°C**

Strain	Mean Lifespan + SEM(Days)	Total N	*p*-value vs. N2	% effect on N2
ctrl	13.1 ± 0.2	307		
pme-1(O/E)	13.9 ± 0.2	460	0.009	6

Data from two independent experiments and multiple bombarded lines are combined. Experiments were carried out at 25°C.

Table AIII.2C

***pme-1* high-copy overexpression lifespan at 20°C**

Strain	Mean Lifespan + SEM(Days)	Total N	*p*-value vs. N2	% effect on N2
ctrl	20.6 ± 0.4	149		
pme-1(O/E)	19.1 ± 0.2	348	<0.001	-7

Data from two independent experiments and multiple microinjected lines are combined. Experiments were carried out at 25°C.

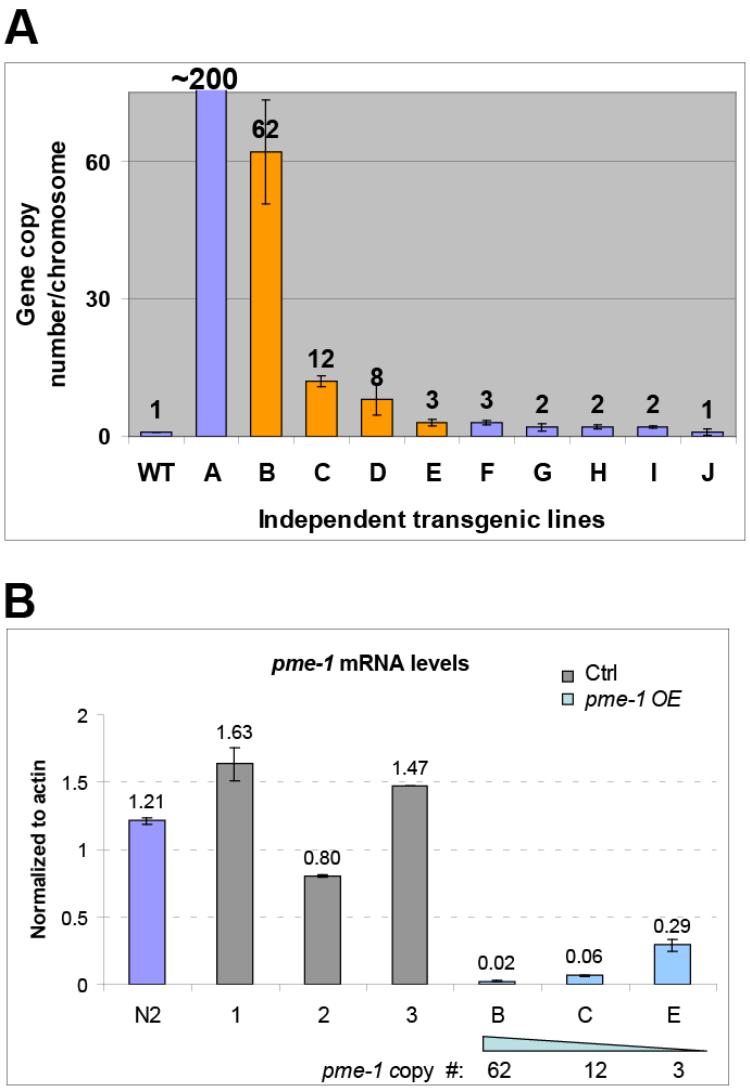


Figure AIII.1. *pme-1* expression is shut down in strains harboring extra copies of genomic *pme-1*. (A) Chromosomal copy number of *pme-1* gene in independent transgenic lines generated by bombardment were quantified using quantitative PCR. The signal from qPCR of *pme-1* gene was normalized to N2 levels. Bars colored orange highlight the lines selected for lifespan analyses. (B) *pme-1* mRNA levels were measured by RT-qPCR and normalized to actin. 1, 2, and 3 represent three indepent lines carrying the control transgene *unc-119(O/E)*, whereas B, C, and E denote three indepent lines carrying extra copies of *pme-1*.

Taken together, our data suggest that depleting PARPs does not contribute to considerable changes in the lifespan of worms. Due to the problems we faced overexpressing the *pme-1* gene, we are unable to draw solid conclusions from our lifespan experiments with PARP overexpression. Thus, we tentatively conclude that PARPs, although functionally crucial for many physiological processes, likely are not major players in longevity determination in *C. elegans.*

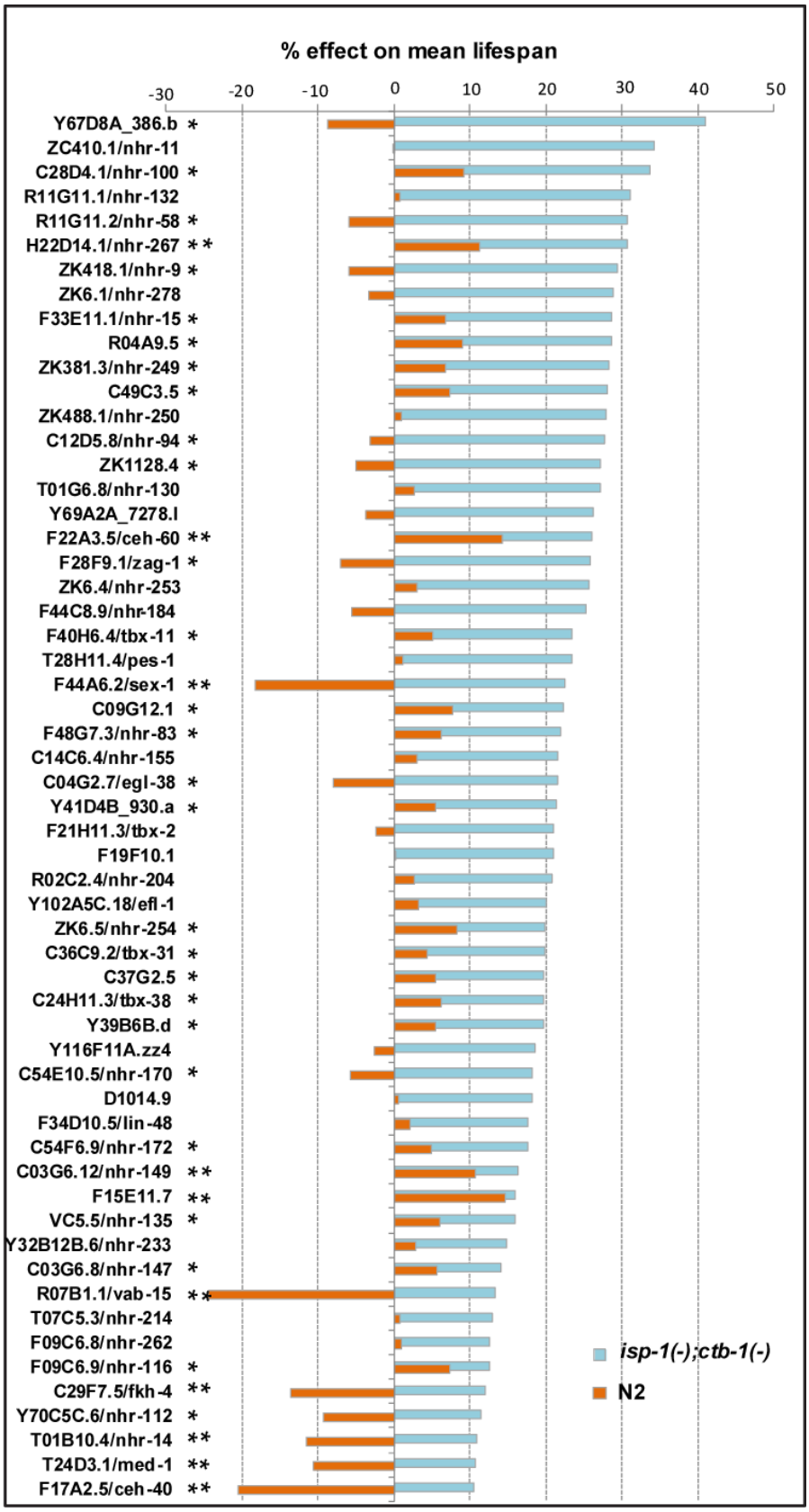
APPENDIX IV

RNAi SCREEN TO TEST THE EFFECTS OF 57 TRANSCRIPTION FACTORS, WHICH ENHANCE THE LONG LIFESPAN OF MITOCHONDRIAL MUTANT *ISP-1;CTB-1*, ON WILD TYPE LONGEVITY

An RNAi screen to identify the transcription factors that mediate the long lifespan of mitochondrial electron transport chain (ETC) mutant *isp-1;ctb-1* have been conducted in our lab (Walter *et al.*, 2011). Although the focus of the screen has been the transcription factors whose knockdown attenuated the prolonged lifespan of *isp-1;ctb-1* mutants, 57 factors which further enhanced the ETC mutant longevity also emerged (Ludivine Walter, unpublished results). We reasoned that the factors which further extended ETC mutant lifespan may represent transcriptional modulators of aging in general. To test this hypothesis, we conducted a small scale RNAi screen testing the effects of inactivating each transcription factor in the N2 wild type background. We found 22 factors that significantly extended N2 lifespan when knocked down, 4 of which more than 10%. Interestingly, 13 transcription factors moderately shortened N2 lifespan where 6 factors among them resulted in a greater than 10% lifespan reduction (Figure AIV.1).

Therefore, our RNAi screen uncovered a total of 35 putative transcriptional regulators with an impact on *C. elegans* longevity. Future experiments to confirm my initial results and characterize the longevity roles of candidate transcription factors will help identify novel longevity determinants and pathways in worms.

Figure AIV.1. The effect on N2 lifespan of depleting 57 ETC-lifespan extending transcription factors. The percentage effect of each RNAi treatment on the mean lifespan of N2+ctrl and *isp-1(-);ctb-1(-)*+ctrl is plotted. All clones were tested once on N2 except C28D4.1/*nhr-100,* H22D14.1*/nhr-267,* F22A3.5*/ceh-60,* C03G6.12*/nhr-149,* and F15E11.7 were tested twice, where data pooled from two independent repeats are displayed. *denotes *p*-value<0.05 compared the N2+ctrl mean lifespan, ** denotes an effect on N2+ctrl lifespan more than 10%. RNAi expressing bacteria were grown overnight, concentrated to OD 5.0, seeded on RNAi plates containing 4mM IPTG. RNAi was initiated at embryo stage and the experiments performed at 20°C. Worms were refed on Day 6 of adulthood to avoid food deprivation. Survival was scored every other day. Kaplan Meier Survival analysis and log-rank statistics were used to compute mean lifespans and *p*-values.


% effect on mean lifespan
-30
-20
-10
0
10
20
30
40
50
Y67D8A_386.b *
ZC410.1/nhr-11
C28D4.1/nhr-100 *
R11G11.1/nhr-132
R11G11.2/nhr-58 *
H22D14.1/nhr-267 **
ZK418.1/nhr-9 *
ZK6.1/nhr-278
F33E11.1/nhr-15 *
R04A9.5 *
ZK381.3/nhr-249 *
C49C3.5 *
ZK488.1/nhr-250
C12D5.8/nhr-94 *
ZK1128.4 *
T01G6.8/nhr-130
Y69A2A_7278.l
F22A3.5/ceh-60 **
F28F9.1/zag-1 *
ZK6.4/nhr-253
F44C8.9/nhr-184
F40H6.4/tbx-11 *
T28H11.4/pes-1
F44A6.2/sex-1 **
C09G12.1 *
F48G7.3/nhr-83 *
C14C6.4/nhr-155
C04G2.7/egl-38 *
Y41D4B_930.a *
F21H11.3/tbx-2
F19F10.1
R02C2.4/nhr-204
Y102A5C.18/efl-1
ZK6.5/nhr-254 *
C36C9.2/tbx-31 *
C37G2.5 *
C24H11.3/tbx-38 *
Y39B6B.d *
Y116F11A.zz4
C54E10.5/nhr-170 *
D1014.9
F34D10.5/lin-48
C54F6.9/nhr-172 *
C03G6.12/nhr-149 **
F15E11.7 **
VC5.5/nhr-135 *
Y32B12B.6/nhr-233
C03G6.8/nhr-147 *
R07B1.1/vab-15 **
T07C5.3/nhr-214
F09C6.8/nhr-262
F09C6.9/nhr-116 *
C29F7.5/fkh-4 **
Y70C5C.6/nhr-112 *
T01B10.4/nhr-14 **
T24D3.1/med-1 **
F17A2.5/ceh-40 **
isp-1(-);ctb-1(-)
N2

REFERENCES

Ahn, S. H., Cheung, W. L., Hsu, J. Y., Diaz, R. L., Smith, M. M. and Allis, C. D. (2005). Sterile 20 kinase phosphorylates histone H2B at serine 10 during hydrogen peroxide-induced apoptosis in S. cerevisiae. *Cell* 120(1): 25-36.

Ajuh, P. M., Browne, G. J., Hawkes, N. A., Cohen, P. T., Roberts, S. G. and Lamond, A. I. (2000). Association of a protein phosphatase 1 activity with the human factor C1 (HCF) complex. *Nucleic Acids Res* 28(3): 678-86.

Akerfelt, M., Morimoto, R. I. and Sistonen, L. (2010). Heat shock factors: integrators of cell stress, development and lifespan. *Nat Rev Mol Cell Biol* 11(8): 545-55.

Albert, P. S., Brown, S. J. and Riddle, D. L. (1981). Sensory control of dauer larva formation in Caenorhabditis elegans. *J Comp Neurol* 198(3): 435-51.

An, J. H. and Blackwell, T. K. (2003). SKN-1 links C. elegans mesendodermal specification to a conserved oxidative stress response. *Genes Dev* 17(15): 1882-93.

An, J. H., Vranas, K., Lucke, M., Inoue, H., Hisamoto, N., Matsumoto, K. and Blackwell, T. K. (2005). Regulation of the Caenorhabditis elegans oxidative stress defense protein SKN-1 by glycogen synthase kinase-3. *Proc Natl Acad Sci U S A* 102(45): 16275-80.

Arantes-Oliveira, N., Apfeld, J., Dillin, A. and Kenyon, C. (2002). Regulation of life-span by germ-line stem cells in Caenorhabditis elegans. *Science* 295(5554): 502-5.

Arden, K. C. (2008). FOXO animal models reveal a variety of diverse roles for FOXO transcription factors. *Oncogene* 27(16): 2345-50.

Bai, P., Canto, C., Brunyanszki, A., Huber, A., Szanto, M., Cen, Y., Yamamoto, H., Houten, S. M., Kiss, B., Oudart, H., Gergely, P., Menissier-de Murcia, J., Schreiber, V., Sauve, A. A. and Auwerx, J. PARP-2 regulates SIRT1 expression and whole-body energy expenditure. *Cell Metab* 13(4): 450-60.

Bai, P., Canto, C., Oudart, H., Brunyanszki, A., Cen, Y., Thomas, C., Yamamoto, H., Huber, A., Kiss, B., Houtkooper, R. H., Schoonjans, K., Schreiber, V., Sauve, A. A., Menissier-de Murcia, J. and Auwerx, J. PARP-1 inhibition increases mitochondrial metabolism through SIRT1 activation. *Cell Metab* 13(4): 461-8.

Banks, A. S., Kon, N., Knight, C., Matsumoto, M., Gutierrez-Juarez, R., Rossetti, L., Gu, W. and Accili, D. (2008). SirT1 gain of function increases energy efficiency and prevents diabetes in mice. *Cell Metab* 8(4): 333-41.

Barger, J. L., Kayo, T., Vann, J. M., Arias, E. B., Wang, J., Hacker, T. A., Wang, Y., Raederstorff, D., Morrow, J. D., Leeuwenburgh, C., Allison, D. B., Saupe, K. W., Cartee, G. D., Weindruch, R. and Prolla, T. A. (2008). A low dose of dietary resveratrol partially mimics caloric restriction and retards aging parameters in mice. *PLoS One* 3(6): e2264.

Bass, T. M., Weinkove, D., Houthoofd, K., Gems, D. and Partridge, L. (2007). Effects of resveratrol on lifespan in Drosophila melanogaster and Caenorhabditis elegans. *Mech Ageing Dev* 128(10): 546-52.

Beitel, G. J., Tuck, S., Greenwald, I. and Horvitz, H. R. (1995). The Caenorhabditis elegans gene lin-1 encodes an ETS-domain protein and defines a branch of the vulval induction pathway. *Genes Dev* 9(24): 3149-62.

Berdichevsky, A., Viswanathan, M., Horvitz, H. R. and Guarente, L. (2006). C. elegans SIR-2.1 interacts with 14-3-3 proteins to activate DAF-16 and extend life span. *Cell* 125(6): 1165-77.

Berman, J. R. and Kenyon, C. (2006). Germ-cell loss extends C. elegans life span through regulation of DAF-16 by kri-1 and lipophilic-hormone signaling. *Cell* 124(5): 1055-68.

Bishop, N. A. and Guarente, L. (2007). Two neurons mediate diet-restriction-induced longevity in C. elegans. *Nature* 447(7144): 545-9.

Blackwell, T. K., Bowerman, B., Priess, J. R. and Weintraub, H. (1994). Formation of a monomeric DNA binding domain by Skn-1 bZIP and homeodomain elements. *Science* 266(5185): 621-8.

Boily, G., Seifert, E. L., Bevilacqua, L., He, X. H., Sabourin, G., Estey, C., Moffat, C., Crawford, S., Saliba, S., Jardine, K., Xuan, J., Evans, M., Harper, M. E. and McBurney, M. W. (2008). SirT1 regulates energy metabolism and response to caloric restriction in mice. *PLoS One* 3(3): e1759.

Bordone, L., Cohen, D., Robinson, A., Motta, M. C., van Veen, E., Czopik, A., Steele, A. D., Crowe, H., Marmor, S., Luo, J., Gu, W. and Guarente, L. (2007). SIRT1 transgenic mice show phenotypes resembling calorie restriction. *Aging Cell* 6(6): 759-67.

Boura, E., Silhan, J., Herman, P., Vecer, J., Sulc, M., Teisinger, J., Obsilova, V. and Obsil, T. (2007). Both the N-terminal loop and wing W2 of the forkhead domain of transcription factor Foxo4 are important for DNA binding. *J Biol Chem* 282(11): 8265-75.

Bowerman, B., Draper, B. W., Mello, C. C. and Priess, J. R. (1993). The maternal gene skn-1 encodes a protein that is distributed unequally in early C. elegans embryos. *Cell* 74(3): 443-52.

Bowerman, B., Eaton, B. A. and Priess, J. R. (1992). skn-1, a maternally expressed gene required to specify the fate of ventral blastomeres in the early C. elegans embryo. *Cell* 68(6): 1061-75.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics* 77(1): 71-94.

Breslow, N. E. and Clayton, D. G. (1993). Approximate Inference in Generalized Linear Mixed Models. *Journal of the American Statistical Association* 88(421): 9-25.

Brunet, A., Kanai, F., Stehn, J., Xu, J., Sarbassova, D., Frangioni, J. V., Dalal, S. N., DeCaprio, J. A., Greenberg, M. E. and Yaffe, M. B. (2002). 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. *J Cell Biol* 156(5): 817-28.

Brunet, A., Sweeney, L. B., Sturgill, J. F., Chua, K. F., Greer, P. L., Lin, Y., Tran, H., Ross, S. E., Mostoslavsky, R., Cohen, H. Y., Hu, L. S., Cheng, H. L., Jedrychowski, M. P., Gygi, S. P., Sinclair, D. A., Alt, F. W. and Greenberg, M. E. (2004). Stress-dependent

regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* 303(5666): 2011-5.

Budovskaya, Y. V., Wu, K., Southworth, L. K., Jiang, M., Tedesco, P., Johnson, T. E. and Kim, S. K. (2008). An elt-3/elt-5/elt-6 GATA transcription circuit guides aging in C. elegans. *Cell* 134(2): 291-303.

Burgering, B. M. and Kops, G. J. (2002). Cell cycle and death control: long live Forkheads. *Trends Biochem Sci* 27(7): 352-60.

Burkle, A., Diefenbach, J., Brabeck, C. and Beneke, S. (2005). Ageing and PARP. *Pharmacol Res* 52(1): 93-9.

Cahill, C. M., Tzivion, G., Nasrin, N., Ogg, S., Dore, J., Ruvkun, G. and Alexander-Bridges, M. (2001). Phosphatidylinositol 3-kinase signaling inhibits DAF-16 DNA binding and function via 14-3-3-dependent and 14-3-3-independent pathways. *J Biol Chem* 276(16): 13402-10.

Cai, Y., Jin, J., Swanson, S. K., Cole, M. D., Choi, S. H., Florens, L., Washburn, M. P., Conaway, J. W. and Conaway, R. C. Subunit composition and substrate specificity of a MOF-containing histone acetyltransferase distinct from the male-specific lethal (MSL) complex. *J Biol Chem* 285(7): 4268-72.

Chan, J. Y., Han, X. L. and Kan, Y. W. (1993). Cloning of Nrf1, an NF-E2-related transcription factor, by genetic selection in yeast. *Proc Natl Acad Sci U S A* 90(23): 11371-5.

Cheeseman, I. M., Niessen, S., Anderson, S., Hyndman, F., Yates, J. R., 3rd, Oegema, K. and Desai, A. (2004). A conserved protein network controls assembly of the outer kinetochore and its ability to sustain tension. *Genes Dev* 18(18): 2255-68.

Chen, D. and Guarente, L. (2007). SIR2: a potential target for calorie restriction mimetics. *Trends Mol Med* 13(2): 64-71.

Chen, D., Steele, A. D., Lindquist, S. and Guarente, L. (2005). Increase in activity during calorie restriction requires Sirt1. *Science* 310(5754): 1641.

Chen, H., Nandi, A. K., Li, X. and Bieberich, C. J. (2002). NKX-3.1 interacts with prostate-derived Ets factor and regulates the activity of the PSA promoter. *Cancer Res* 62(2): 338-40.

Chen, S., Whetstine, J. R., Ghosh, S., Hanover, J. A., Gali, R. R., Grosu, P. and Shi, Y. (2009). The conserved NAD(H)-dependent corepressor CTBP-1 regulates Caenorhabditis elegans life span. *Proc Natl Acad Sci U S A* 106(5): 1496-501.

Chinnadurai, G. (2002). CtBP, an unconventional transcriptional corepressor in development and oncogenesis. *Mol Cell* 9(2): 213-24.

Chinnadurai, G. (2003). CtBP family proteins: more than transcriptional corepressors. *Bioessays* 25(1): 9-12.

Chinnadurai, G. (2009). The transcriptional corepressor CtBP: a foe of multiple tumor suppressors. *Cancer Res* 69(3): 731-4.

Choe, K. P., Przybysz, A. J. and Strange, K. (2009). The WD40 repeat protein WDR-23

functions with the CUL4/DDB1 ubiquitin ligase to regulate nuclear abundance and activity of SKN-1 in Caenorhabditis elegans. *Mol Cell Biol* 29(10): 2704-15.

Chowdhury, D., Xu, X., Zhong, X., Ahmed, F., Zhong, J., Liao, J., Dykxhoorn, D. M., Weinstock, D. M., Pfeifer, G. P. and Lieberman, J. (2008). A PP4-phosphatase complex dephosphorylates gamma-H2AX generated during DNA replication. *Mol Cell* 31(1): 33-46.

Crooks, G. E., Hon, G., Chandonia, J. M. and Brenner, S. E. (2004). WebLogo: a sequence logo generator. *Genome Res* 14(6): 1188-90.

Curran, S. P., Wu, X., Riedel, C. G. and Ruvkun, G. (2009). A soma-to-germline transformation in long-lived Caenorhabditis elegans mutants. *Nature* 459(7250): 1079-84.

Daitoku, H., Hatta, M., Matsuzaki, H., Aratani, S., Ohshima, T., Miyagishi, M., Nakajima, T. and Fukamizu, A. (2004). Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. *Proc Natl Acad Sci U S A* 101(27): 10042-7.

Davis, R. J. (2000). Signal transduction by the JNK group of MAP kinases. *Cell* 103(2): 239-52.

Dejosez, M., Krumenacker, J. S., Zitur, L. J., Passeri, M., Chu, L. F., Songyang, Z., Thomson, J. A. and Zwaka, T. P. (2008). Ronin is essential for embryogenesis and the pluripotency of mouse embryonic stem cells. *Cell* 133(7): 1162-74.

Dennis, G., Jr., Sherman, B. T., Hosack, D. A., Yang, J., Gao, W., Lane, H. C. and Lempicki, R. A. (2003). DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 4(5): P3.

Donmez, G. and Guarente, L. (2010). Aging and disease: connections to sirtuins. *Aging Cell* 9(2): 285-90.

Dorman, J. B., Albinder, B., Shroyer, T. and Kenyon, C. (1995). The age-1 and daf-2 genes function in a common pathway to control the lifespan of Caenorhabditis elegans. *Genetics* 141(4): 1399-406.

Durocher, D., Taylor, I. A., Sarbassova, D., Haire, L. F., Westcott, S. L., Jackson, S. P., Smerdon, S. J. and Yaffe, M. B. (2000). The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. *Mol Cell* 6(5): 1169-82.

Eisen, M. B., Spellman, P. T., Brown, P. O. and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 95(25): 14863-8.

Eisenmann, D. M. (2005). Wnt signaling. *WormBook*: 1-17.

Eisenmann, D. M., Maloof, J. N., Simske, J. S., Kenyon, C. and Kim, S. K. (1998). The beta-catenin homolog BAR-1 and LET-60 Ras coordinately regulate the Hox gene lin-39 during Caenorhabditis elegans vulval development. *Development* 125(18): 3667-80.

Essers, M. A., de Vries-Smits, L. M., Barker, N., Polderman, P. E., Burgering, B. M. and Korswagen, H. C. (2005). Functional interaction between beta-catenin and FOXO in

oxidative stress signaling. *Science* 308(5725): 1181-4.

Feldman, R. J., Sementchenko, V. I., Gayed, M., Fraig, M. M. and Watson, D. K. (2003). Pdef expression in human breast cancer is correlated with invasive potential and altered gene expression. *Cancer Res* 63(15): 4626-31.

Ferguson, E. L. and Horvitz, H. R. (1985). Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode Caenorhabditis elegans. *Genetics* 110(1): 17-72.

Freiman, R. N. and Herr, W. (1997). Viral mimicry: common mode of association with HCF by VP16 and the cellular protein LZIP. *Genes Dev* 11(23): 3122-7.

Furuyama, T., Nakazawa, T., Nakano, I. and Mori, N. (2000). Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J* 349(Pt 2): 629-34.

Garigan, D., Hsu, A. L., Fraser, A. G., Kamath, R. S., Ahringer, J. and Kenyon, C. (2002). Genetic analysis of tissue aging in Caenorhabditis elegans: a role for heat-shock factor and bacterial proliferation. *Genetics* 161(3): 1101-12.

George, K. M., Leonard, M. W., Roth, M. E., Lieuw, K. H., Kioussis, D., Grosveld, F. and Engel, J. D. (1994). Embryonic expression and cloning of the murine GATA-3 gene. *Development* 120(9): 2673-86.

Gerster, T. and Roeder, R. G. (1988). A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. *Proc Natl Acad Sci U S A* 85(17): 6347-51.

Ghazi, A., Henis-Korenblit, S. and Kenyon, C. (2009). A transcription elongation factor that links signals from the reproductive system to lifespan extension in Caenorhabditis elegans. *PLoS Genet* 5(9): e1000639.

Giannakou, M. E., Goss, M., Junger, M. A., Hafen, E., Leevers, S. J. and Partridge, L. (2004). Long-lived Drosophila with overexpressed dFOXO in adult fat body. *Science* 305(5682): 361.

Giannakou, M. E. and Partridge, L. (2004). The interaction between FOXO and SIRT1: tipping the balance towards survival. *Trends Cell Biol* 14(8): 408-12.

Gingras, A. C., Caballero, M., Zarske, M., Sanchez, A., Hazbun, T. R., Fields, S., Sonenberg, N., Hafen, E., Raught, B. and Aebersold, R. (2005). A novel, evolutionarily conserved protein phosphatase complex involved in cisplatin sensitivity. *Mol Cell Proteomics* 4(11): 1725-40.

Goto, H., Motomura, S., Wilson, A. C., Freiman, R. N., Nakabeppu, Y., Fukushima, K., Fujishima, M., Herr, W. and Nishimoto, T. (1997). A single-point mutation in HCF causes temperature-sensitive cell-cycle arrest and disrupts VP16 function. *Genes Dev* 11(6): 726-37.

Gottlieb, S. and Ruvkun, G. (1994). daf-2, daf-16 and daf-23: genetically interacting genes controlling Dauer formation in Caenorhabditis elegans. *Genetics* 137(1): 107-20.

Graves, B. J. and Petersen, J. M. (1998). Specificity within the ets family of transcription factors. *Adv Cancer Res* 75: 1-55.

Greer, E. L. and Brunet, A. (2009). Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in C. elegans. *Aging Cell* 8(2): 113-27.

Gregorieff, A., Stange, D. E., Kujala, P., Begthel, H., van den Born, M., Korving, J., Peters, P. J. and Clevers, H. (2009). The ets-domain transcription factor Spdef promotes maturation of goblet and paneth cells in the intestinal epithelium. *Gastroenterology* 137(4): 1333-45 e1-3.

Grube, K. and Burkle, A. (1992). Poly(ADP-ribose) polymerase activity in mononuclear leukocytes of 13 mammalian species correlates with species-specific life span. *Proc Natl Acad Sci U S A* 89(24): 11759-63.

Gu, X., Zerbini, L. F., Otu, H. H., Bhasin, M., Yang, Q., Joseph, M. G., Grall, F., Onatunde, T., Correa, R. G. and Libermann, T. A. (2007). Reduced PDEF expression increases invasion and expression of mesenchymal genes in prostate cancer cells. *Cancer Res* 67(9): 4219-26.

Gunawardane, R. N., Sgroi, D. C., Wrobel, C. N., Koh, E., Daley, G. Q. and Brugge, J. S. (2005). Novel role for PDEF in epithelial cell migration and invasion. *Cancer Res* 65(24): 11572-80.

Gunther, M., Laithier, M. and Brison, O. (2000). A set of proteins interacting with transcription factor Sp1 identified in a two-hybrid screening. *Mol Cell Biochem* 210(1-2): 131-42.

Gupta, S., Stamatoyannopoulos, J. A., Bailey, T. L. and Noble, W. S. (2007). Quantifying similarity between motifs. *Genome Biol* 8(2): R24.

Halaschek-Wiener, J., Khattra, J. S., McKay, S., Pouzyrev, A., Stott, J. M., Yang, G. S., Holt, R. A., Jones, S. J., Marra, M. A., Brooks-Wilson, A. R. and Riddle, D. L. (2005). Analysis of long-lived C. elegans daf-2 mutants using serial analysis of gene expression. *Genome Res* 15(5): 603-15.

Hamilton, B., Dong, Y., Shindo, M., Liu, W., Odell, I., Ruvkun, G. and Lee, S. S. (2005). A systematic RNAi screen for longevity genes in C. elegans. *Genes Dev* 19(13): 1544-55.

Han, M. and Sternberg, P. W. (1990). let-60, a gene that specifies cell fates during C. elegans vulval induction, encodes a ras protein. *Cell* 63(5): 921-31.

Hart, A. H., Reventar, R. and Bernstein, A. (2000). Genetic analysis of ETS genes in C. elegans. *Oncogene* 19(55): 6400-8.

Harvey, K. F., Pfleger, C. M. and Hariharan, I. K. (2003). The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* 114(4): 457-67.

Hassan, H. M. and Fridovich, I. (1979). Intracellular production of superoxide radical and of hydrogen peroxide by redox active compounds. *Arch Biochem Biophys* 196(2): 385-95.

Henderson, S. T. and Johnson, T. E. (2001). daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. *Curr*

Biol 11(24): 1975-80.

Hertweck, M., Gobel, C. and Baumeister, R. (2004). C. elegans SGK-1 is the critical component in the Akt/PKB kinase complex to control stress response and life span. *Dev Cell* 6(4): 577-88.

Hildebrand, J. D. and Soriano, P. (2002). Overlapping and unique roles for C-terminal binding protein 1 (CtBP1) and CtBP2 during mouse development. *Mol Cell Biol* 22(15): 5296-307.

Honda, Y. and Honda, S. (1999). The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. *FASEB J* 13(11): 1385-93.

Honda, Y. and Honda, S. (2002). Oxidative stress and life span determination in the nematode Caenorhabditis elegans. *Ann N Y Acad Sci* 959: 466-74.

Hsin, H. and Kenyon, C. (1999). Signals from the reproductive system regulate the lifespan of C. elegans. *Nature* 399(6734): 362-6.

Hsu, A. L., Murphy, C. T. and Kenyon, C. (2003). Regulation of aging and age-related disease by DAF-16 and heat-shock factor. *Science* 300(5622): 1142-5.

Huang da, W., Sherman, B. T. and Lempicki, R. A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4(1): 44-57.

Huang, H. and Tindall, D. J. (2007). Dynamic FoxO transcription factors. *J Cell Sci* 120(Pt 15): 2479-87.

Hwangbo, D. S., Gershman, B., Tu, M. P., Palmer, M. and Tatar, M. (2004). Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. *Nature* 429(6991): 562-6.

Inoue, H., Hisamoto, N., An, J. H., Oliveira, R. P., Nishida, E., Blackwell, T. K. and Matsumoto, K. (2005). The C. elegans p38 MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response. *Genes Dev* 19(19): 2278-83.

Ivy, J. M., Klar, A. J. and Hicks, J. B. (1986). Cloning and characterization of four SIR genes of Saccharomyces cerevisiae. *Mol Cell Biol* 6(2): 688-702.

Izeta, A., Malcomber, S. and O'Hare, P. (2003a). Primary structure and compartmentalization of Drosophila melanogaster host cell factor. *Gene* 305(2): 175-83.

Izeta, A., Malcomber, S., O'Rourke, D., Hodgkin, J. and O'Hare, P. (2003b). A C-terminal targeting signal controls differential compartmentalisation of Caenorhabditis elegans host cell factor (HCF) to the nucleus or mitochondria. *Eur J Cell Biol* 82(10): 495-504.

Jedlicka, P. and Gutierrez-Hartmann, A. (2008). Ets transcription factors in intestinal morphogenesis, homeostasis and disease. *Histol Histopathol* 23(11): 1417-24.

Jedlicka, P., Mortin, M. A. and Wu, C. (1997). Multiple functions of Drosophila heat shock transcription factor in vivo. *Embo J* 16(9): 2452-62.

Johnson, K. M., Mahajan, S. S. and Wilson, A. C. (1999). Herpes simplex virus transactivator VP16 discriminates between HCF-1 and a novel family member, HCF-2. *J Virol* 73(5): 3930-40.

Jomova, K., Vondrakova, D., Lawson, M. and Valko, M. (2010). Metals, oxidative stress and neurodegenerative disorders. *Mol Cell Biochem* 345(1-2): 91-104.

Julien, E. and Herr, W. (2003). Proteolytic processing is necessary to separate and ensure proper cell growth and cytokinesis functions of HCF-1. *EMBO J* 22(10): 2360-9.

Julien, E. and Herr, W. (2004). A switch in mitotic histone H4 lysine 20 methylation status is linked to M phase defects upon loss of HCF-1. *Mol Cell* 14(6): 713-25.

Kaeberlein, M., Kirkland, K. T., Fields, S. and Kennedy, B. K. (2004). Sir2-independent life span extension by calorie restriction in yeast. *PLoS Biol* 2(9): E296.

Kaeberlein, M., McVey, M. and Guarente, L. (1999). The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. *Genes Dev* 13(19): 2570-80.

Kaeberlein, M. and Powers, R. W., 3rd (2007). Sir2 and calorie restriction in yeast: a skeptical perspective. *Ageing Res Rev* 6(2): 128-40.

Kahn, N. W., Rea, S. L., Moyle, S., Kell, A. and Johnson, T. E. (2008). Proteasomal dysfunction activates the transcription factor SKN-1 and produces a selective oxidative-stress response in Caenorhabditis elegans. *Biochem J* 409(1): 205-13.

Kajimura, S., Seale, P., Tomaru, T., Erdjument-Bromage, H., Cooper, M. P., Ruas, J. L., Chin, S., Tempst, P., Lazar, M. A. and Spiegelman, B. M. (2008). Regulation of the brown and white fat gene programs through a PRDM16/CtBP transcriptional complex. *Genes Dev* 22(10): 1397-409.

Kappeler, L., De Magalhaes Filho, C., Dupont, J., Leneuve, P., Cervera, P., Perin, L., Loudes, C., Blaise, A., Klein, R., Epelbaum, J., Le Bouc, Y. and Holzenberger, M. (2008). Brain IGF-1 receptors control mammalian growth and lifespan through a neuroendocrine mechanism. *PLoS Biol* 6(10): e254.

Karpac, J. and Jasper, H. (2009). Insulin and JNK: optimizing metabolic homeostasis and lifespan. *Trends Endocrinol Metab* 20(3): 100-6.

Katan, M., Haigh, A., Verrijzer, C. P., van der Vliet, P. C. and O'Hare, P. (1990). Characterization of a cellular factor which interacts functionally with Oct-1 in the assembly of a multicomponent transcription complex. *Nucleic Acids Res* 18(23): 6871-80.

Kenyon, C., Chang, J., Gensch, E., Rudner, A. and Tabtiang, R. (1993). A C. elegans mutant that lives twice as long as wild type. *Nature* 366(6454): 461-4.

Kenyon, C. J. (2010). The genetics of ageing. *Nature* 464(7288): 504-12.

Kim, M. Y., Zhang, T. and Kraus, W. L. (2005). Poly(ADP-ribosyl)ation by PARP-1: 'PAR-laying' NAD+ into a nuclear signal. *Genes Dev* 19(17): 1951-67.

Kittler, R., Putz, G., Pelletier, L., Poser, I., Heninger, A. K., Drechsel, D., Fischer, S., Konstantinova, I., Habermann, B., Grabner, H., Yaspo, M. L., Himmelbauer, H.,

Korn, B., Neugebauer, K., Pisabarro, M. T. and Buchholz, F. (2004). An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. *Nature* 432(7020): 1036-40.

Knez, J., Piluso, D., Bilan, P. and Capone, J. P. (2006). Host cell factor-1 and E2F4 interact via multiple determinants in each protein. *Mol Cell Biochem* 288(1-2): 79-90.

Kobayashi, A., Ito, E., Toki, T., Kogame, K., Takahashi, S., Igarashi, K., Hayashi, N. and Yamamoto, M. (1999). Molecular cloning and functional characterization of a new Cap'n' collar family transcription factor Nrf3. *J Biol Chem* 274(10): 6443-52.

Kobayashi, M. and Yamamoto, M. (2006). Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Adv Enzyme Regul* 46: 113-40.

Koschmieder, S., Halmos, B., Levantini, E. and Tenen, D. G. (2009). Dysregulation of the C/EBPalpha differentiation pathway in human cancer. *J Clin Oncol* 27(4): 619-28.

Krishnakumar, R. and Kraus, W. L. The PARP side of the nucleus: molecular actions, physiological outcomes, and clinical targets. *Mol Cell* 39(1): 8-24.

Kristie, T. M., LeBowitz, J. H. and Sharp, P. A. (1989). The octamer-binding proteins form multi-protein--DNA complexes with the HSV alpha TIF regulatory protein. *Embo J* 8(13): 4229-38.

Kristie, T. M., Pomerantz, J. L., Twomey, T. C., Parent, S. A. and Sharp, P. A. (1995). The cellular C1 factor of the herpes simplex virus enhancer complex is a family of polypeptides. *J Biol Chem* 270(9): 4387-94.

Kwon, E. S., Narasimhan, S. D., Yen, K. and Tissenbaum, H. A. (2010). A new DAF-16 isoform regulates longevity. *Nature* 466(7305): 498-502.

Lee, R. Y., Hench, J. and Ruvkun, G. (2001). Regulation of C. elegans DAF-16 and its human ortholog FKHRL1 by the daf-2 insulin-like signaling pathway. *Curr Biol* 11(24): 1950-7.

Lee, S. and Herr, W. (2001). Stabilization but not the transcriptional activity of herpes simplex virus VP16-induced complexes is evolutionarily conserved among HCF family members. *J Virol* 75(24): 12402-11.

Lee, S., Horn, V., Julien, E., Liu, Y., Wysocka, J., Bowerman, B., Hengartner, M. O. and Herr, W. (2007). Epigenetic regulation of histone H3 serine 10 phosphorylation status by HCF-1 proteins in C. elegans and mammalian cells. *PLoS One* 2(11): e1213.

Lee, S. S., Kennedy, S., Tolonen, A. C. and Ruvkun, G. (2003). DAF-16 target genes that control C. elegans life-span and metabolism. *Science* 300(5619): 644-7.

Lehtinen, M. K., Yuan, Z., Boag, P. R., Yang, Y., Villen, J., Becker, E. B., DiBacco, S., de la Iglesia, N., Gygi, S., Blackwell, T. K. and Bonni, A. (2006). A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. *Cell* 125(5): 987-1001.

Li, J., Ebata, A., Dong, Y., Rizki, G., Iwata, T. and Lee, S. S. (2008). Caenorhabditis elegans HCF-1 functions in longevity maintenance as a DAF-16 regulator. *PLoS Biol* 6(9): e233.

Li, J., Tewari, M., Vidal, M. and Lee, S. S. (2007). The 14-3-3 protein FTT-2 regulates DAF-16 in Caenorhabditis elegans. *Dev Biol* 301(1): 82-91.

Li, Y., Wang, W. J., Cao, H., Lu, J., Wu, C., Hu, F. Y., Guo, J., Zhao, L., Yang, F., Zhang, Y. X., Li, W., Zheng, G. Y., Cui, H., Chen, X., Zhu, Z., He, H., Dong, B., Mo, X., Zeng, Y. and Tian, X. L. (2009). Genetic association of FOXO1A and FOXO3A with longevity trait in Han Chinese populations. *Hum Mol Genet* 18(24): 4897-904.

Libert, S., Chao, Y., Zwiener, J. and Pletcher, S. D. (2008). Realized immune response is enhanced in long-lived puc and chico mutants but is unaffected by dietary restriction. *Mol Immunol* 45(3): 810-7.

Libina, N., Berman, J. R. and Kenyon, C. (2003). Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan. *Cell* 115(4): 489-502.

Lillevali, K., Matilainen, T., Karis, A. and Salminen, M. (2004). Partially overlapping expression of Gata2 and Gata3 during inner ear development. *Dev Dyn* 231(4): 775-81.

Lin, J., Puigserver, P., Donovan, J., Tarr, P. and Spiegelman, B. M. (2002). Peroxisome proliferator-activated receptor gamma coactivator 1beta (PGC-1beta), a novel PGC-1-related transcription coactivator associated with host cell factor. *J Biol Chem* 277(3): 1645-8.

Lin, K., Dorman, J. B., Rodan, A. and Kenyon, C. (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science* 278(5341): 1319-22.

Lin, K., Hsin, H., Libina, N. and Kenyon, C. (2001). Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nat Genet* 28(2): 139-45.

Lin, S. J., Defossez, P. A. and Guarente, L. (2000). Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. *Science* 289(5487): 2126-8.

Link, C. D., Cypser, J. R., Johnson, C. J. and Johnson, T. E. (1999). Direct observation of stress response in Caenorhabditis elegans using a reporter transgene. *Cell Stress Chaperones* 4(4): 235-42.

Liu, X., Brutlag, D. L. and Liu, J. S. (2001). BioProspector: discovering conserved DNA motifs in upstream regulatory regions of co-expressed genes. *Pac Symp Biocomput*: 127-38.

Lu, R. and Misra, V. (2000). Zhangfei: a second cellular protein interacts with herpes simplex virus accessory factor HCF in a manner similar to Luman and VP16. *Nucleic Acids Res* 28(12): 2446-54.

Lu, R., Yang, P., O'Hare, P. and Misra, V. (1997). Luman, a new member of the CREB/ATF family, binds to herpes simplex virus VP16-associated host cellular factor. *Mol Cell Biol* 17(9): 5117-26.

Luo, X. and Kraus, W. L. A one and a two ... expanding roles for poly(ADP-ribose) polymerases in metabolism. *Cell Metab* 13(4): 353-5.

Lyu, J., Jho, E. H. and Lu, W. (2011). Smek promotes histone deacetylation to suppress transcription of Wnt target gene brachyury in pluripotent embryonic stem cells. *Cell Res*.

Maduro, M. F., Meneghini, M. D., Bowerman, B., Broitman-Maduro, G. and Rothman, J. H. (2001). Restriction of mesendoderm to a single blastomere by the combined action of SKN-1 and a GSK-3beta homolog is mediated by MED-1 and -2 in C. elegans. *Mol Cell* 7(3): 475-85.

Mallo, G. V., Kurz, C. L., Couillault, C., Pujol, N., Granjeaud, S., Kohara, Y. and Ewbank, J. J. (2002). Inducible antibacterial defense system in C. elegans. *Curr Biol* 12(14): 1209-14.

Maroulakou, I. G. and Bowe, D. B. (2000). Expression and function of Ets transcription factors in mammalian development: a regulatory network. *Oncogene* 19(55): 6432-42.

Mathews, W. R., Guido, D. M., Fisher, M. A. and Jaeschke, H. (1994). Lipid peroxidation as molecular mechanism of liver cell injury during reperfusion after ischemia. *Free Radic Biol Med* 16(6): 763-70.

McElwee, J., Bubb, K. and Thomas, J. H. (2003). Transcriptional outputs of the Caenorhabditis elegans forkhead protein DAF-16. *Aging Cell* 2(2): 111-21.

McElwee, J. J., Schuster, E., Blanc, E., Piper, M. D., Thomas, J. H., Patel, D. S., Selman, C., Withers, D. J., Thornton, J. M., Partridge, L. and Gems, D. (2007). Evolutionary conservation of regulated longevity assurance mechanisms. *Genome Biol* 8(7): R132.

Mendoza, M. C., Du, F., Iranfar, N., Tang, N., Ma, H., Loomis, W. F. and Firtel, R. A. (2005). Loss of SMEK, a novel, conserved protein, suppresses MEK1 null cell polarity, chemotaxis, and gene expression defects. *Mol Cell Biol* 25(17): 7839-53.

Mihindukulasuriya, K. A., Zhou, G., Qin, J. and Tan, T. H. (2004). Protein phosphatase 4 interacts with and down-regulates insulin receptor substrate 4 following tumor necrosis factor-alpha stimulation. *J Biol Chem* 279(45): 46588-94.

Misra, V., Rapin, N., Akhova, O., Bainbridge, M. and Korchinski, P. (2005). Zhangfei is a potent and specific inhibitor of the host cell factor-binding transcription factor Luman. *J Biol Chem* 280(15): 15257-66.

Moazed, B., Banman, S. L., Wilkinson, G. A. and Roesler, W. J. (2011). TCERG1 inhibits C/EBPalpha through a mechanism that does not involve sequestration of C/EBPalpha at pericentromeric heterochromatin. *J Cell Biochem*.

Mohler, J., Vani, K., Leung, S. and Epstein, A. (1991). Segmentally restricted, cephalic expression of a leucine zipper gene during Drosophila embryogenesis. *Mech Dev* 34(1): 3-9.

Moi, P., Chan, K., Asunis, I., Cao, A. and Kan, Y. W. (1994). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc Natl Acad Sci U S A* 91(21): 9926-30.

Molavi, B. and Mehta, J. L. (2004). Oxidative stress in cardiovascular disease: molecular basis of its deleterious effects, its detection, and therapeutic considerations. *Curr Opin*

Cardiol 19(5): 488-93.

Morimoto, R. I. (2008). Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. *Genes Dev* 22(11): 1427-38.

Morley, J. F., Brignull, H. R., Weyers, J. J. and Morimoto, R. I. (2002). The threshold for polyglutamine-expansion protein aggregation and cellular toxicity is dynamic and influenced by aging in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* 99(16): 10417-22.

Morley, J. F. and Morimoto, R. I. (2004). Regulation of longevity in Caenorhabditis elegans by heat shock factor and molecular chaperones. *Mol Biol Cell* 15(2): 657-64.

Morris, J. Z., Tissenbaum, H. A. and Ruvkun, G. (1996). A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans. *Nature* 382(6591): 536-9.

Motta, M. C., Divecha, N., Lemieux, M., Kamel, C., Chen, D., Gu, W., Bultsma, Y., McBurney, M. and Guarente, L. (2004). Mammalian SIRT1 represses forkhead transcription factors. *Cell* 116(4): 551-63.

Mourtada-Maarabouni, M., Kirkham, L., Jenkins, B., Rayner, J., Gonda, T. J., Starr, R., Trayner, I., Farzaneh, F. and Williams, G. T. (2003). Functional expression cloning reveals proapoptotic role for protein phosphatase 4. *Cell Death Differ* 10(9): 1016-24.

Mukhopadhyay, A., Oh, S. W. and Tissenbaum, H. A. (2006). Worming pathways to and from DAF-16/FOXO. *Exp Gerontol* 41(10): 928-34.

Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Kamath, R. S., Ahringer, J., Li, H. and Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. *Nature* 424(6946): 277-83.

Nardelli, J., Thiesson, D., Fujiwara, Y., Tsai, F. Y. and Orkin, S. H. (1999). Expression and genetic interaction of transcription factors GATA-2 and GATA-3 during development of the mouse central nervous system. *Dev Biol* 210(2): 305-21.

Neumann-Haefelin, E., Qi, W., Finkbeiner, E., Walz, G., Baumeister, R. and Hertweck, M. (2008). SHC-1/p52Shc targets the insulin/IGF-1 and JNK signaling pathways to modulate life span and stress response in C. elegans. *Genes Dev* 22(19): 2721-35.

Nguyen, T., Sherratt, P. J. and Pickett, C. B. (2003). Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annu Rev Pharmacol Toxicol* 43: 233-60.

Nicholas, H. R., Lowry, J. A., Wu, T. and Crossley, M. (2008). The Caenorhabditis elegans protein CTBP-1 defines a new group of THAP domain-containing CtBP corepressors. *J Mol Biol* 375(1): 1-11.

Nye, J. A., Petersen, J. M., Gunther, C. V., Jonsen, M. D. and Graves, B. J. (1992). Interaction of murine ets-1 with GGA-binding sites establishes the ETS domain as a new DNA-binding motif. *Genes Dev* 6(6): 975-90.

Oberdoerffer, P., Michan, S., McVay, M., Mostoslavsky, R., Vann, J., Park, S. K., Hartlerode, A., Stegmuller, J., Hafner, A., Loerch, P., Wright, S. M., Mills, K. D., Bonni, A., Yankner, B. A., Scully, R., Prolla, T. A., Alt, F. W. and Sinclair, D. A. (2008). SIRT1

redistribution on chromatin promotes genomic stability but alters gene expression during aging. *Cell* 135(5): 907-18.

Obsil, T., Ghirlando, R., Anderson, D. E., Hickman, A. B. and Dyda, F. (2003). Two 14-3-3 binding motifs are required for stable association of Forkhead transcription factor FOXO4 with 14-3-3 proteins and inhibition of DNA binding. *Biochemistry* 42(51): 15264-72.

Oettgen, P., Finger, E., Sun, Z., Akbarali, Y., Thamrongsak, U., Boltax, J., Grall, F., Dube, A., Weiss, A., Brown, L., Quinn, G., Kas, K., Endress, G., Kunsch, C. and Libermann, T. A. (2000). PDEF, a novel prostate epithelium-specific ets transcription factor, interacts with the androgen receptor and activates prostate-specific antigen gene expression. *J Biol Chem* 275(2): 1216-25.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G. I., Lee, L., Tissenbaum, H. A. and Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature* 389(6654): 994-9.

Oh, S. W., Mukhopadhyay, A., Svrzikapa, N., Jiang, F., Davis, R. J. and Tissenbaum, H. A. (2005). JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. *Proc Natl Acad Sci U S A* 102(12): 4494-9.

Ohtsuka, H., Azuma, K., Murakami, H. and Aiba, H. (2011). hsf1 (+) extends chronological lifespan through Ecl1 family genes in fission yeast. *Mol Genet Genomics* 285(1): 67-77.

Oliveira, R. P., Porter Abate, J., Dilks, K., Landis, J., Ashraf, J., Murphy, C. T. and Blackwell, T. K. (2009). Condition-adapted stress and longevity gene regulation by Caenorhabditis elegans SKN-1/Nrf. *Aging Cell* 8(5): 524-41.

Paradis, S., Ailion, M., Toker, A., Thomas, J. H. and Ruvkun, G. (1999). A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans. *Genes Dev* 13(11): 1438-52.

Paradis, S. and Ruvkun, G. (1998). Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. *Genes Dev* 12(16): 2488-98.

Pasco, M. Y., Catoire, H., Parker, J. A., Brais, B., Rouleau, G. A. and Neri, C. (2010). Cross-talk between canonical Wnt signaling and the sirtuin-FoxO longevity pathway to protect against muscular pathology induced by mutant PABPN1 expression in C. elegans. *Neurobiol Dis* 38(3): 425-33.

Pfluger, P. T., Herranz, D., Velasco-Miguel, S., Serrano, M. and Tschop, M. H. (2008). Sirt1 protects against high-fat diet-induced metabolic damage. *Proc Natl Acad Sci U S A* 105(28): 9793-8.

Pillai, J. B., Isbatan, A., Imai, S. and Gupta, M. P. (2005). Poly(ADP-ribose) polymerase-1-dependent cardiac myocyte cell death during heart failure is mediated by NAD+ depletion and reduced Sir2alpha deacetylase activity. *J Biol Chem* 280(52): 43121-30.

Piluso, D., Bilan, P. and Capone, J. P. (2002). Host cell factor-1 interacts with and antagonizes transactivation by the cell cycle regulatory factor Miz-1. *J Biol Chem*

277(48): 46799-808.

Pinkston-Gosse, J. and Kenyon, C. (2007). DAF-16/FOXO targets genes that regulate tumor growth in Caenorhabditis elegans. *Nat Genet*.

Pinkston, J. M., Garigan, D., Hansen, M. and Kenyon, C. (2006). Mutations that increase the life span of C. elegans inhibit tumor growth. *Science* 313(5789): 971-5.

Pleiss, J. A., Whitworth, G. B., Bergkessel, M. and Guthrie, C. (2007). Transcript specificity in yeast pre-mRNA splicing revealed by mutations in core spliceosomal components. *PLoS Biol* 5(4): e90.

Rains, J. L. and Jain, S. K. (2011). Oxidative stress, insulin signaling, and diabetes. *Free Radic Biol Med* 50(5): 567-75.

Reilly, P. T. and Herr, W. (2002). Spontaneous reversion of tsBN67 cell proliferation and cytokinesis defects in the absence of HCF-1 function. *Exp Cell Res* 277(1): 119-30.

Reuter, S., Gupta, S. C., Chaturvedi, M. M. and Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med* 49(11): 1603-16.

Rimann, I. and Hajnal, A. (2007). Regulation of anchor cell invasion and uterine cell fates by the egl-43 Evi-1 proto-oncogene in Caenorhabditis elegans. *Dev Biol* 308(1): 187-95.

Rine, J. and Herskowitz, I. (1987). Four genes responsible for a position effect on expression from HML and HMR in Saccharomyces cerevisiae. *Genetics* 116(1): 9-22.

Rogina, B. and Helfand, S. L. (2004). Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc Natl Acad Sci U S A* 101(45): 15998-6003.

Scarr, R. B. and Sharp, P. A. (2002). PDCD2 is a negative regulator of HCF-1 (C1). *Oncogene* 21(34): 5245-54.

Schmid, C., Schwarz, V. and Hutter, H. (2006). AST-1, a novel ETS-box transcription factor, controls axon guidance and pharynx development in C. elegans. *Dev Biol* 293(2): 403-13.

Sementchenko, V. I. and Watson, D. K. (2000). Ets target genes: past, present and future. *Oncogene* 19(55): 6533-48.

Seth, A. and Watson, D. K. (2005). ETS transcription factors and their emerging roles in human cancer. *Eur J Cancer* 41(16): 2462-78.

Shaw, W. M., Luo, S., Landis, J., Ashraf, J. and Murphy, C. T. (2007). The C. elegans TGF-beta Dauer pathway regulates longevity via insulin signaling. *Curr Biol* 17(19): 1635-45.

Shi, Y., Sawada, J., Sui, G., Affar el, B., Whetstine, J. R., Lan, F., Ogawa, H., Luke, M. P. and Nakatani, Y. (2003). Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* 422(6933): 735-8.

Simmer, F., Tijsterman, M., Parrish, S., Koushika, S. P., Nonet, M. L., Fire, A., Ahringer, J. and Plasterk, R. H. (2002). Loss of the putative RNA-directed RNA polymerase RRF-3 makes C. elegans hypersensitive to RNAi. *Curr Biol* 12(15): 1317-9.

Singh, V. and Aballay, A. (2006). Heat-shock transcription factor (HSF)-1 pathway required for Caenorhabditis elegans immunity. *Proc Natl Acad Sci U S A* 103(35): 13092-7.

Smith, J. J., Kenney, R. D., Gagne, D. J., Frushour, B. P., Ladd, W., Galonek, H. L., Israelian, K., Song, J., Razvadauskaite, G., Lynch, A. V., Carney, D. P., Johnson, R. J., Lavu, S., Iffland, A., Elliott, P. J., Lambert, P. D., Elliston, K. O., Jirousek, M. R., Milne, J. C. and Boss, O. (2009). Small molecule activators of SIRT1 replicate signaling pathways triggered by calorie restriction in vivo. *BMC Syst Biol* 3: 31.

Sousa-Nunes, R., Chia, W. and Somers, W. G. (2009). Protein phosphatase 4 mediates localization of the Miranda complex during Drosophila neuroblast asymmetric divisions. *Genes Dev* 23(3): 359-72.

Spradling, A. C., Stern, D., Beaton, A., Rhem, E. J., Laverty, T., Mozden, N., Misra, S. and Rubin, G. M. (1999). The Berkeley Drosophila Genome Project gene disruption project: Single P-element insertions mutating 25% of vital Drosophila genes. *Genetics* 153(1): 135-77.

Stainier, D. Y. (2002). A glimpse into the molecular entrails of endoderm formation. *Genes Dev* 16(8): 893-907.

Sue, N., Jack, B. H., Eaton, S. A., Pearson, R. C., Funnell, A. P., Turner, J., Czolij, R., Denyer, G., Bao, S., Molero-Navajas, J. C., Perkins, A., Fujiwara, Y., Orkin, S. H., Bell-Anderson, K. and Crossley, M. (2008). Targeted disruption of the basic Kruppel-like factor gene (Klf3) reveals a role in adipogenesis. *Mol Cell Biol* 28(12): 3967-78.

Swindell, W. R., Masternak, M. M., Kopchick, J. J., Conover, C. A., Bartke, A. and Miller, R. A. (2009). Endocrine regulation of heat shock protein mRNA levels in long-lived dwarf mice. *Mech Ageing Dev* 130(6): 393-400.

Sykiotis, G. P. and Bohmann, D. (2008). Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. *Dev Cell* 14(1): 76-85.

Sykiotis, G. P. and Bohmann, D. (2010). Stress-activated cap'n'collar transcription factors in aging and human disease. *Sci Signal* 3(112): re3.

Tabb, D. L., McDonald, W. H. and Yates, J. R., 3rd (2002). DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. *J Proteome Res* 1(1): 21-6.

Tatar, M. (2003). Unearthing Loci that influence life span. *Sci Aging Knowledge Environ* 2003(9): PE5.

Tatar, M., Khazaeli, A. A. and Curtsinger, J. W. (1997). Chaperoning extended life. *Nature* 390(6655): 30.

Thomas-Chollier, M., Sand, O., Turatsinze, J. V., Janky, R., Defrance, M., Vervisch, E., Brohee, S. and van Helden, J. (2008). RSAT: regulatory sequence analysis tools. *Nucleic Acids Res* 36(Web Server issue): W119-27.

Thyagarajan, B., Blaszczak, A. G., Chandler, K. J., Watts, J. L., Johnson, W. E. and Graves, B. J. (2010). ETS-4 is a transcriptional regulator of life span in Caenorhabditis elegans. *PLoS Genet* 6(9).

Tissenbaum, H. A. and Guarente, L. (2001). Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. *Nature* 410(6825): 227-30.

Tissenbaum, H. A. and Ruvkun, G. (1998). An insulin-like signaling pathway affects both

longevity and reproduction in Caenorhabditis elegans. *Genetics* 148(2): 703-17.

Troemel, E. R., Chu, S. W., Reinke, V., Lee, S. S., Ausubel, F. M. and Kim, D. H. (2006). p38 MAPK regulates expression of immune response genes and contributes to longevity in C. elegans. *PLoS Genet* 2(11): e183.

Tullet, J. M., Hertweck, M., An, J. H., Baker, J., Hwang, J. Y., Liu, S., Oliveira, R. P., Baumeister, R. and Blackwell, T. K. (2008). Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in C. elegans. *Cell* 132(6): 1025-38.

Turner, D. P., Moussa, O., Sauane, M., Fisher, P. B. and Watson, D. K. (2007). Prostate-derived ETS factor is a mediator of metastatic potential through the inhibition of migration and invasion in breast cancer. *Cancer Res* 67(4): 1618-25.

Tusher, V. G., Tibshirani, R. and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A* 98(9): 5116-21.

Tyagi, S., Chabes, A. L., Wysocka, J. and Herr, W. (2007). E2F activation of S phase promoters via association with HCF-1 and the MLL family of histone H3K4 methyltransferases. *Mol Cell* 27(1): 107-19.

Tyagi, S. and Herr, W. (2009). E2F1 mediates DNA damage and apoptosis through HCF-1 and the MLL family of histone methyltransferases. *EMBO J* 28(20): 3185-95.

Tzivion, G., Shen, Y. H. and Zhu, J. (2001). 14-3-3 proteins; bringing new definitions to scaffolding. *Oncogene* 20(44): 6331-8.

van der Horst, A. and Burgering, B. M. (2007). Stressing the role of FoxO proteins in lifespan and disease. *Nat Rev Mol Cell Biol* 8(6): 440-50.

van der Horst, A., Tertoolen, L. G., de Vries-Smits, L. M., Frye, R. A., Medema, R. H. and Burgering, B. M. (2004). FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). *J Biol Chem* 279(28): 28873-9.

Viger, R. S., Guittot, S. M., Anttonen, M., Wilson, D. B. and Heikinheimo, M. (2008). Role of the GATA family of transcription factors in endocrine development, function, and disease. *Mol Endocrinol* 22(4): 781-98.

Viswanathan, M., Kim, S. K., Berdichevsky, A. and Guarente, L. (2005). A role for SIR-2.1 regulation of ER stress response genes in determining C. elegans life span. *Dev Cell* 9(5): 605-15.

Vogel, J. L. and Kristie, T. M. (2000). The novel coactivator C1 (HCF) coordinates multiprotein enhancer formation and mediates transcription activation by GABP. *Embo J* 19(4): 683-90.

Walker, G. A. and Lithgow, G. J. (2003). Lifespan extension in C. elegans by a molecular chaperone dependent upon insulin-like signals. *Aging Cell* 2(2): 131-9.

Walter, L., Baruah, A., Chang, H. W., Pace, H. and Lee, S. S. (2011). The Homeobox Protein CEH-23 Mediates Prolonged Longevity in Response to Impaired Mitochondrial Electron Transport Chain in *C. elegans*. *PLoS Biology, in press*.

Wang, J., Robida-Stubbs, S., Tullet, J. M., Rual, J. F., Vidal, M. and Blackwell, T. K. (2010). RNAi screening implicates a SKN-1-dependent transcriptional response in stress

resistance and longevity deriving from translation inhibition. *PLoS Genet* 6(8).

Wang, M. C., Bohmann, D. and Jasper, H. (2003). JNK signaling confers tolerance to oxidative stress and extends lifespan in Drosophila. *Dev Cell* 5(5): 811-6.

Wang, M. C., Bohmann, D. and Jasper, H. (2005). JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling. *Cell* 121(1): 115-25.

Wang, M. C., O'Rourke, E. J. and Ruvkun, G. (2008). Fat metabolism links germline stem cells and longevity in C. elegans. *Science* 322(5903): 957-60.

Wang, W. and Shakes, D. C. (1997). Expression patterns and transcript processing of ftt-1 and ftt-2, two C. elegans 14-3-3 homologues. *J Mol Biol* 268(3): 619-30.

Wang, Y., Oh, S. W., Deplancke, B., Luo, J., Walhout, A. J. and Tissenbaum, H. A. (2006). C. elegans 14-3-3 proteins regulate life span and interact with SIR-2.1 and DAF-16/FOXO. *Mech Ageing Dev* 127(9): 741-7.

Wang, Y. and Tissenbaum, H. A. (2006). Overlapping and distinct functions for a Caenorhabditis elegans SIR2 and DAF-16/FOXO. *Mech Ageing Dev* 127(1): 48-56.

Wang, Z., Pandey, A. and Hart, G. W. (2007). Dynamic interplay between O-linked N-acetylglucosaminylation and glycogen synthase kinase-3-dependent phosphorylation. *Mol Cell Proteomics* 6(8): 1365-79.

Weiss, M. J. and Orkin, S. H. (1995). GATA transcription factors: key regulators of hematopoiesis. *Exp Hematol* 23(2): 99-107.

Willcox, B. J., Donlon, T. A., He, Q., Chen, R., Grove, J. S., Yano, K., Masaki, K. H., Willcox, D. C., Rodriguez, B. and Curb, J. D. (2008). FOXO3A genotype is strongly associated with human longevity. *Proc Natl Acad Sci U S A* 105(37): 13987-92.

Wilson, A. C., Boutros, M., Johnson, K. M. and Herr, W. (2000). HCF-1 amino- and carboxy-terminal subunit association through two separate sets of interaction modules: involvement of fibronectin type 3 repeats. *Mol Cell Biol* 20(18): 6721-30.

Wolff, S., Ma, H., Burch, D., Maciel, G. A., Hunter, T. and Dillin, A. (2006). SMK-1, an essential regulator of DAF-16-mediated longevity. *Cell* 124(5): 1039-53.

Wood, J. G., Rogina, B., Lavu, S., Howitz, K., Helfand, S. L., Tatar, M. and Sinclair, D. (2004). Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* 430(7000): 686-9.

Wu, H. I., Brown, J. A., Dorie, M. J., Lazzeroni, L. and Brown, J. M. (2004). Genome-wide identification of genes conferring resistance to the anticancer agents cisplatin, oxaliplatin, and mitomycin C. *Cancer Res* 64(11): 3940-8.

Wysocka, J. and Herr, W. (2003). The herpes simplex virus VP16-induced complex: the makings of a regulatory switch. *Trends Biochem Sci* 28(6): 294-304.

Wysocka, J., Liu, Y., Kobayashi, R. and Herr, W. (2001). Developmental and cell-cycle regulation of Caenorhabditis elegans HCF phosphorylation. *Biochemistry* 40(19): 5786-94.

Wysocka, J., Myers, M. P., Laherty, C. D., Eisenman, R. N. and Herr, W. (2003). Human

Sin3 deacetylase and trithorax-related Set1/Ash2 histone H3-K4 methyltransferase are tethered together selectively by the cell-proliferation factor HCF-1. *Genes Dev* 17(7): 896-911.

Xiao, P. and Capone, J. P. (1990). A cellular factor binds to the herpes simplex virus type 1 transactivator Vmw65 and is required for Vmw65-dependent protein-DNA complex assembly with Oct-1. *Mol Cell Biol* 10(9): 4974-7.

Xu, C., Li, C. Y. and Kong, A. N. (2005). Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res* 28(3): 249-68.

Yang, Y., Hou, H., Haller, E. M., Nicosia, S. V. and Bai, W. (2005). Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation. *EMBO J* 24(5): 1021-32.

Yokoyama, A., Wang, Z., Wysocka, J., Sanyal, M., Aufiero, D. J., Kitabayashi, I., Herr, W. and Cleary, M. L. (2004). Leukemia proto-oncoprotein MLL forms a SET1-like histone methyltransferase complex with menin to regulate Hox gene expression. *Mol Cell Biol* 24(13): 5639-49.

Yokoyama, K., Fukumoto, K., Murakami, T., Harada, S., Hosono, R., Wadhwa, R., Mitsui, Y. and Ohkuma, S. (2002). Extended longevity of Caenorhabditis elegans by knocking in extra copies of hsp70F, a homolog of mot-2 (mortalin)/mthsp70/Grp75. *FEBS Lett* 516(1-3): 53-7.

Yoon, Y. S., Lee, M. W., Ryu, D., Kim, J. H., Ma, H., Seo, W. Y., Kim, Y. N., Kim, S. S., Lee, C. H., Hunter, T., Choi, C. S., Montminy, M. R. and Koo, S. H. (2010). Suppressor of MEK null (SMEK)/protein phosphatase 4 catalytic subunit (PP4C) is a key regulator of hepatic gluconeogenesis. *Proc Natl Acad Sci U S A* 107(41): 17704-9.

Yuan, R., Tsaih, S. W., Petkova, S. B., Marin de Evsikova, C., Xing, S., Marion, M. A., Bogue, M. A., Mills, K. D., Peters, L. L., Bult, C. J., Rosen, C. J., Sundberg, J. P., Harrison, D. E., Churchill, G. A. and Paigen, B. (2009). Aging in inbred strains of mice: study design and interim report on median lifespans and circulating IGF1 levels. *Aging Cell* 8(3): 277-87.

Zhang, Q., Piston, D. W. and Goodman, R. H. (2002). Regulation of corepressor function by nuclear NADH. *Science* 295(5561): 1895-7.

Zhou, J., Liu, Y., Zhang, W., Popov, V. M., Wang, M., Pattabiraman, N., Sune, C., Cvekl, A., Wu, K., Jiang, J., Wang, C. and Pestell, R. G. (2010). Transcription elongation regulator 1 is a co-integrator of the cell fate determination factor Dachshund homolog 1. *J Biol Chem* 285(51): 40342-50.